The impact of the intestinal microbiota on microbial infections

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Gewidmet meiner Oma

*Johanna Thiel, *27.07.1927*

Summary

A diverse community of microorganisms that form a complex ecosystem colonizes the gastrointestinal tract. This community has been termed the intestinal microbiota. The microbiota and their metabolites play a crucial role in the physiology of the host. In particular, resident bacteria promote the development and maturation of the immune system, but also protect the host against invading enteric pathogens by a process called colonization resistance. The intestinal microbiota confers resistance directly by producing inhibitory substances and competing for nutrients or indirectly, by strengthening the epithelial barrier and enhancing protective immune-mediated pathways. Disruption of the resident microbiota, e.g. by medical treatment, is linked to higher susceptibility to infections. Moreover, the composition of the microbiota is highly variable among humans and these differences may contribute additionally to varying susceptibilities to enteric pathogens. Until now, little is known about the contributions of individual members of the intestinal microbiota to confer colonization resistance.

To understand how different intestinal microbial communities influence the susceptibility to enteric pathogens, isogenic mouse lines from different breeding facilities were analyzed regarding their microbiota composition. Subsequently, mouse lines were challenged with *Salmonella* Typhimurium (non-typhoidal model) and *Citrobacter rodentium* to identify microbial signatures associated with decreased disease severity.

Analyzing the fecal microbiota by 16S rRNA gene amplicon sequencing revealed that isogenic mouse lines from different breeding facilities featured a distinct microbiota composition. Moreover, isogenic mouse lines displayed different susceptibilities to *S.* Typhimurium infection. Fecal transplantation from resistant to susceptible mice reproduced the protected phenotype. Statistical analysis of 16S rRNA sequencing data identified bacteria of the families S24-7, Prevotellaceae and Verrucomicrobiaceae to be associated with increased resistance. Transfer of 11 bacterial species, cultured from protected mice, in susceptible mice diminished disease severity. Interferon-γ was identified as novel microbiota modulated factor and demonstrated that it is required for conferring resistance through immune-mediated colonization resistance.

In addition, isogenic mouse lines with different microbiota composition varied in their susceptibility to *Citrobacter rodentium* infection. By using cohousing experiments of susceptible and resistance mice coupled with statistical analysis of 16S rRNA sequencing data, two bacteria of the family Lachnospiraceae were identified important to diminish luminal colonization.

The results highlighted that differences in the microbiota of immunocompetent mice influence the outcome of enteric infections. Moreover, novel microbial signatures and mechanisms were identified to confer resistance to *S.* Typhimurium and *C. rodentium* infections. Characterization of microbial interactions and metabolites as well as the corresponding immune factors that influence host physiology are important contributions that may enable developing improved therapies for mucosal infections.

Zusammenfassung

Eine vielfältige Gemeinschaft von Mikroorganismen kolonisiert den Gastrointestinaltrakt und bildet dort ein komplexes Ökosystem. Diese Gemeinschaft wird als Darmmikrobiota bezeichnet und spielt eine entscheidende Rolle in der Physiologie des Wirts. Insbesondere fördern kommensale Bakterien die Entwicklung und Reifung des Immunsystems. Darüber hinaus schützen sie den Wirt vor Krankheitserregern, was auch als Kolonisationsresistenz bezeichnet wird. Die Darmmikrobiota vermittelt dies direkt, indem sie hemmende Substanzen produziert, oder indirekt durch die Stärkung der Epithelbarriere und der Förderung von protektiven Immunantworten. Die Zerstörung der kommensalen Mikrobiota, z.B. durch eine Behandlung mit Medikamenten, geht einher mit einer höheren Anfälligkeit gegenüber Infektionen. Außerdem ist die Zusammensetzung der Mikrobiota bei Menschen variabel, was Unterschiede im Krankheitsverlauf von Enteropathogenen erklären könnte. Bisher ist nur wenig darüber bekannt, ob und wie die einzelnen Mitglieder der Darmmikrobiota zu der Kolonisationsresistenz beitragen.

Um zu verstehen, wie verschiedene mikrobielle Gemeinschaften die Anfälligkeit des Wirts gegenüber Enteropathogenen beeinflussen, wurden isogene Mauslinien verschiedener Züchtungseinrichtungen hinsichtlich ihrer Mikrobiotazusammensetzung analysiert. Anschließend wurden Mauslinien mit *Salmonella* Typhimurium (nicht-typhoides Modell) und *Citrobacter rodentiu*m infiziert, um Bakterien zu identifizieren, die den Schweregrad der Erkrankung positiv beeinflussen.

Die Analyse der Mikrobiota mittels 16S-rRNA-Sequenzierung ergab, dass isogene Mauslinien aus verschiedenen Züchtungseinrichtungen eine unterschiedliche Zusammensetzung der Mikrobiota aufweisen. Außerdem zeigten isogene Mauslinien unterschiedliche Anfälligkeiten gegenüber Infektionen mit *S.* Typhimurium. Eine Stuhltransplantation bildete in anfälligen Mäusen den geschützten Phänotyp nach. Die statistische Analyse von 16S-rRNA-Sequenzierungsdaten zeigte, dass Bakterien der Familien S24-7, Prevotellaceae und Verrucomicrobiaceae mit einer erhöhten Resistenz assoziiert sind. Die Übertragung von 11 bakteriellen Spezies, die aus geschützten Mäusen kultiviert wurden, verminderte bei anfälligen Mäusen die Schwere der Erkrankung. Interferon-γ wurde als neuartiger Mikrobiota-modulierender Faktor identifiziert. Es konnte gezeigt werden, dass Interferon-γ für den Transfer der immun-vermittelten Kolonisationsresistenz erforderlich ist.

Außerdem zeigten isogene Mauslinien, die eine unterschiedliche Zusammensetzung der Mikrobiota aufweisen, eine unterschiedliche Anfälligkeit gegenüber *C. rodentium* Infektion. Unter Verwendung von Cohousing-Experimenten von anfälligen und resistenten Mäusen, die gekoppelt wurden mit einer statistischen Analyse von 16S-rRNA-Seqeuenzierungsdaten, wurden zwei Bakterien der Familie Lachnospiraceae identifiziert, die mit verringerter luminalen Kolonisation assoziiert sind.

Die Ergebnisse dieser Arbeit konnten zeigen, dass Unterschiede in der Mikrobiota den Schweregrad von Darminfektionen bei Mäusen beeinflussen. Zudem wurden neue mikrobielle Signaturen und Mechanismen identifiziert, die mit Resistenzen gegenüber Infektionen mit *S.* Typhimurium und *C. rodentium* assoziiert sind. Die Charakterisierung der mikrobiellen Wechselwirkungen und Metaboliten, sowie die entsprechenden Immunfaktoren, welche die Wirtsphysiologie beeinflussen, sind wesentlich, um verbesserte Therapien für mukosale Infektionen zu ermöglichen.

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General introduction

The gastrointestinal tract harbors a vastly diverse and dense microbial community, termed as microbiota (Ley et al., 2008). Different species of bacteria intimately interact with the gut tissue, contributing significantly to the physiology of the host (Guinane and Cotter, 2013). Especially in the last years, new sequencing techniques contributed greatly to the knowledge about the composition and the functionality of the human microbiome in health and disease (D'Argenio and Salvatore, 2015). Moreover, researchers have shed light on the influence of the complex interactions of the microbiota with the mammalian host, which is a result of coevolution, termed as mammalian holobiont.

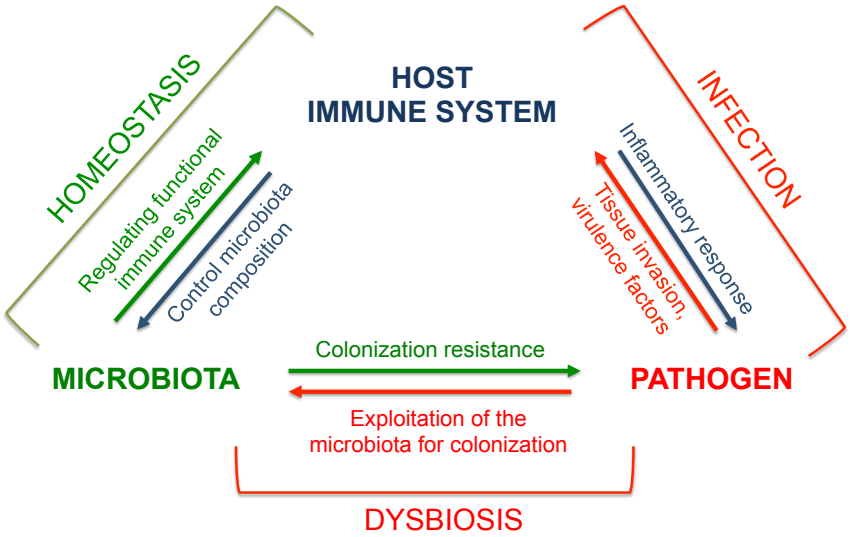

Figure 1.1 - Interplay between the microbiota, host immune system and pathogen. In the intestine, interaction of microbes with host immune cells are crucial for the development of a proper immune system as well as its activation. In turn, cells of the immune system control the microbiota composition contributing to a healthy gut homeostasis. Under homoestatic conditions, the microbiota confer colonization resistance against invading enteric pathogens. However, during dysbiosis enteric pathogens are able to colonize the gastrointestinal tract inducing pathogenicity.

Gut bacteria are important for the digestion of food, the provision of specific nutrients as well as maintaining structural integrity of the gut mucosal barrier, affecting proper development and functioning of the immune system (Belkaid and Hand, 2014; Tremaroli and Bäckhed, 2012). Moreover, intestinal bacteria of the microbiota compete with pathogenic microorganisms, protecting the host against invading pathogens (Bäumler and Sperandio, 2016).

Microbial colonization of the host occurs right after birth and in the first years of life the microbiota increases its diversity (Koenig et al., 2011). Within only 2-5 years an adulthood-like microbiota is formed and each individual develops a relatively distinct, unique at the species level, stable community (Dethlefsen and Relman, 2011; Rodríguez et al., 2015).

Bacteria of the gut microbiota and the immune system contribute together to a balanced homeostasis in the gut by creating a mutual relationship (Figure 1.1). Interaction between the microbiota and the host tissue affect the immune system locally and systemically (Clarke et al., 2010). In turn, host immune cells have large effects on the composition of the gut microbiota (Friman et al., 2002). However, disruption of the intestinal homeostasis leads to dysbiosis, an imbalanced shift of the microbial composition. Dysbiosis is characterized by a decrease of bacterial diversity and results in dysregulation of the host-microbiota interactions. Therefore, dysbiosis augment disease susceptibility to gastrointestinal infections (Brandl et al., 2008; Ivanov et al., 2008; La Cochetière et al., 2008). Alteration of the microbiota composition is caused by a variety of factors such as genetic and environmental factors, i.e. diet, antibiotic treatment and exposure to pathogenic microbes (Round and Mazmanian, 2009). Even among healthy individuals, the diversity of the microbial community varies widely (Human Microbiome Project Consortium, 2012). This could be one reason explaining the huge differences in susceptibility to gastrointestinal infections seen among individuals (Yurist-Doutsch et al., 2014). Experiments with germ-free mice (mice lacking any microbiota) and mice with a reduced microbiota diversity have demonstrated that they are more susceptible to gastrointestinal pathogens (Bohnhoff and Miller, 1962; Inagaki et al., 1996; Reeves et al., 2012). However, it is not fully known yet which commensal bacteria are involved in conferring resistance to infection with enteric pathogens. Moreover, how these commensal bacteria

interact with the pathogen and/or stimulate the immune system to lower susceptibility has only started to be understood. Detailed knowledge about the interactions between the microbiota, the host and invading pathogens would potentially allow the development of new mucosal therapies against enteric infections.

The main aim of this work is to identify bacterial signatures, which correlate with decreased susceptibility to gastrointestinal infections, i.e. to *Salmonella enterica* serovar Typhimurium and *Citrobacter rodentium*. In addition, this work strives to gain more insight into the interplay between the microbiota and the immune system to confer resistance to enteric pathogens.

1.1 The mucosal immune system in the intestine

The gastrointestinal tract is constantly exposed to a vast amount of different antigens, including infectious agents but also harmless materials, i.e. food antigens or commensal bacteria. Cells and molecules of the mucosal immune system support the intestinal barrier function, the physical barrier in the gut. But, antigens can breach the mucosal epithelium and easily be transported across it. Therefore, the mucosal immune system has to control tightly immune responses to these antigens and distinguish innocent materials and potentially harmful agents. In particular, the mucosal immune system has to avoid raising strong immune responses against a broad range of harmless material, whereas it has to induce an effective protective immune response against invading pathogens. How the host organizes these different requirements, will be discussed in the following.

Antigens activate different branches of the mucosal immune system. The mucosal immune system is divided in two parts: the innate and adaptive immune system. The innate immune system is composed of cells and proteins that are ready to mobilize and therefore, able to provide an immediate, but non-specific response to infectious agents. The innate response activates the adaptive immune system including two different lymphocytes, T- and B-Lymphocytes. Unlike the innate immune response, the adaptive immune response is highly specific to a particular pathogen and creates long-lasting immunological responses. Both, the innate and adaptive

immunity contribute with the microbiota to intestinal homeostasis and are crucial for conferring colonization resistance against pathogens.

As already mentioned, the mucosal immune system encounters a wide range of antigens. Therefore, the intestinal epithelial cells (IEC) have to provide a degree of permeability, so that the uptake of essential nutrients and fluids is guaranteed. In turn, IECs have to separate the underlying mucosal tissue from potential pathogens. Additionally, the barrier function of IECs is supported by secretory glycoproteins produced by goblet cells, which form a viscous layer on the intestinal surface, known as mucus layer. In the small intestine, the mucus layer consists of a single layer, but it is composed of two layers in the stomach and colon. The dense inner mucus is firmly attached to the apical epithelial cells and is largely sterile; whereas the looser outer layer tolerates endogenous bacteria, which use mucus as carbon source (Johansson et al., 2011; 2008; Li et al., 2015). Besides mucus production, intestinal epithelial cells (IEC) support barrier function by producing substances, which are microbicidal or prevent microbial growth such as lysozymes and antimicrobial peptides (e.g. defensins, cathelicidins, histatins) (Muniz et al., 2012). Strikingly, the largest fraction of immune cells of the body is found throughout the intestine, localized interspersed with IECs or immediately below IECs in the lamina propria (LP). Immune cells can be found scattered in the tissue and organized in lymphoid structures, such as Peyer's patches (PP), isolated lymphoid follicles (ILFs) and cryptopatches. In particular, PPs and ILFs are specialized to uptake antigens from the lumen via microfold cells (M cells) by transcytosis (Gebert et al., 2004; Owen and Jones, 1974) (Figure 1.2). M cells are specialized epithelial cells, which lack the ability to secrete mucus and enzyme, though are more accessible than enterocytes as they transport large volumes of luminal content via pinocytosis (Mabbott et al., 2013).

After invading the mucosal tissue, bacterial antigens encounter cells of the innate immune system. Cellular compartment of the innate immune system includes common innate immune cells such as myeloid cells (e.g. DCs, macrophages, eosinophils, neutrophils, mast cells) as well as innate lymphoid cells (ILCs). Myeloid cells are able to kill bacteria, e.g. via extracellular nets or via phagocytosis, and activate the adaptive immune system by cytokine

release and antigen presentation. ILCs belong to the lymphoid lineage and express a cytokine profile similar to cells of the adaptive immune system. However, ILCs display the characteristics of innate immune cells such as the lack to generate immunological memory and their activation in early phase of immunity (Klose and Artis, 2016).

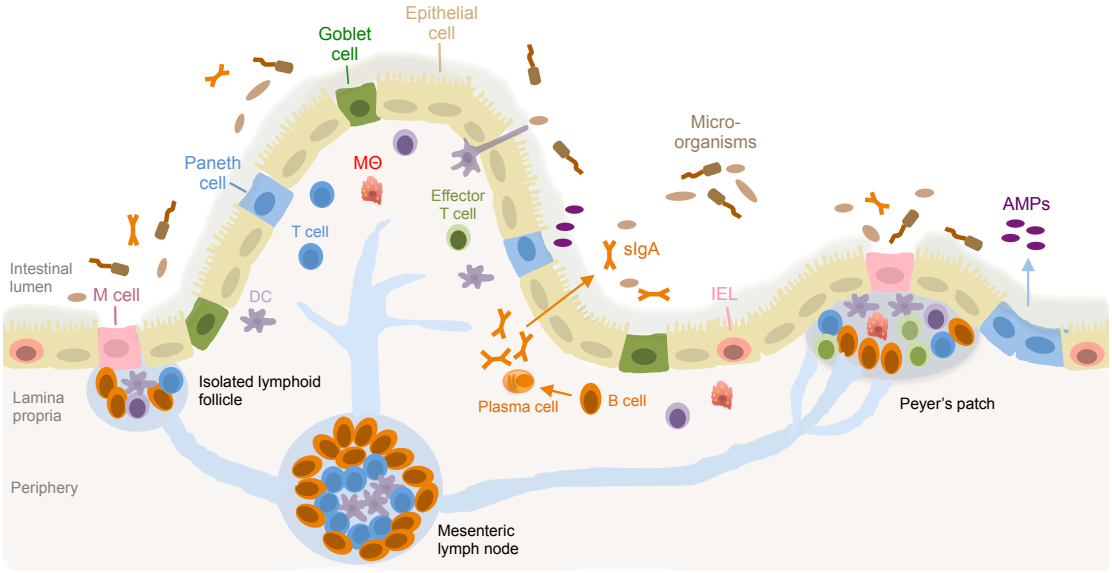

Figure 1.2 – Organization of the gut associated lymphoid tissue (GALT). The intestinal epithelium separates the external luminal environment from the body. Beneath intestinal epithelia cells, the lamina propria harbors organized lymphoid follicles (Peyer's patches and isolated lymphoid follicles), which consist of B and T cells as well as resident and migratory myeloid cells such as dendritic cells. M cells enable antigen transport form the lumen to the lamina propria, activating immune cells in the lymphoid follicles, but also scattered immune cells in the lamina propria. In turn, immune cells migrate to the mesenteric lymph node via lymphatic vessels. In the lamina propria, mature B cells generate IgA, which is secreted in the lumen. AMPs – antimicrobial peptides, DC – dendritic cells, IEL – intestinal epithelial lymphocytes, M cell – microfold cell, MΘ – macrophages, sIgA – secreted immunoglobulin A

Myeloid and epithelial cells sense microorganisms through germline-encoded molecules, known as pattern-recognition receptors (PRRs), which serve as a continuous surveillance system for the host. PRRs recognize microorganisms via conserved microbial structures, so called pathogen-associated molecular patterns (PAMPs). A vast variety of molecules derived from microorganisms function as PAMPs including nucleic acids, proteins, glycans, lipids and lipoproteins, The most studied PRRs are Toll-like receptors (TLRs), nucleotide-binding oligomerization (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (Rig I)-like receptors (RLR) and C-type lectin receptors (Medzhitov, 2007). PRR signaling is critical for inducing an immune response upon recognition of PAMPs. In contrast to pathogens, commensal bacteria of

the microbiota do not trigger inflammatory signaling pathways, but contribute to a normal immune function, which is also mediated by PRRs (Kubinak and Round, 2012). PAMPs are not only restricted to pathogens, rather than present in all microorganisms. Therefore, it was proposed to replace the term PAMPs in absence of infections to microbe-associated molecular pattern (MAMPs) (Mackey and McFall, 2006).

After recognizing bacteria by PRRs innate immune cells also activate adaptive immune responses. DCs take up antigens that crossed the epithelial barrier and PAMPs to initiate a maturation program. Harmless antigens derived from food or commensal bacteria do not elicit a systemic immune response, but provide tolerance; whereas pathogenic bacteria induce a strong immune response. Commensal bacteria do not possess any virulence factors and therefore, do not induce inflammation. $CD103^+$ DCs localized in the mucosa recognize harmless antigens and transport antigens to the draining mesenteric lymph nodes, where they prime a gut-homing phenotype to naive T cells (Iwata et al., 2004). After DCs encounter $CD4^+$ T cells, generation of forkhead box protein P3 (FoxP3) positive regulatory T cells (T_{reg}) is induced (Pabst et al., 2007; Sun et al., 2007). $CD103^+$ DCs also initiate class switching of intestinal B cells to IgA (Uematsu et al., 2008). T_{reg} cells and IgA-expressing B cells recirculate back to the LP where they undergo secondary expansion and ensure local tolerance to harmless antigens. T_{reg} cells produce IL-10 and TGF-β, which suppress pro-inflammatory T cell subsets, including T_H1 and T_H17 cells (Harrison and Powrie, 2013). Mucosal IgA, secreted in the gut lumen, can neutralize bacteria and toxins, averting tissue adhering and induction of an immune response. In addition, IgA also acts in the LP, where IgA binds to bacteria, which crossed the epithelial barrier (Mantis et al., 2011). In both cases IgA avoids stimulation of the immune system against harmless commensal bacteria. Therefore, IgA and T_{reg} cells play a key role in inducing tolerance to harmless antigens.

In contrast to commensal bacteria, pathogenic bacteria possess different virulence factors, which induce a strong inflammatory response in the intestine by activating different immune pathways. Upon detection by PPRs, pathogens elicit production of pro-inflammatory cytokines and attract different immune cells such as neutrophils to the site of the infection. Crossing the

epithelial barrier, intracellular pathogenic bacteria infect phagocytes, which enter the bloodstream, allowing the pathogen to gain access to systemic organs (Monack et al., 2004). Moreover, pathogenic bacteria drive the development of pro-inflammatory cell subsets, such as T_H1 and T_H17 cells, which are important for combat disease (Damsker et al., 2010).

Traditionally, pathogenic bacteria are distinct from commensal bacteria due to the expression of virulence factors. However, it becomes more and more clear that some commensal bacteria share characteristics similar as virulence factors in pathogens (Ayres, 2016). Moreover, some commensal bacteria have been identified to elicit effector cell differentiation, such as T_H1 and T_H17 cells without inducing inflammation and protecting the host to infections (Asseman et al., 2003; Ivanov et al., 2009).

In summary, the mucosal immune system has to distinguish constantly between infectious and harmless material to maintain homeostasis in the gut as well as to rapidly combat enteric infections. How the mucosal immune system differentiates between pathogens and commensal bacteria is important to understand the complex microbe-host interaction, but has still not been fully understood.

1.2 The microbiota regulates mucosal immune responses

The coevolution between the host and the gut microbiota has created a relationship of mutual benefit. The host offers bacteria of the microbiota an essential environment (e.g. niches, nutrients) to exist, whereas the microbiota composition influences strongly the physiology of the host by modeling the mucosal immune system. In turn, genetic deficiencies in important immune pathways also affect the composition of the microbiota.

In the following, I will discuss recent insights into the influence of intestinal bacteria on the mucosal immune system.

1.2.1 Impact of the microbiota on the innate immune system

The microbiota has a pivotal role in the maturation of innate immune cells. Germ-free mice show decreased numbers of several peripheral innate

immune cell populations; including macrophages, neutrophils and monocytes (Khosravi et al., 2014). It has been reported that commensal bacteria are needed for hematopoietic stem cell (HSC)- and also embryo-derived myeloid cell development (Khosravi et al., 2014). Interestingly, fecal transplantation of a complex microbiota composition into germ-free mice restores defects in myeloid cell development (Khosravi et al., 2014). Specifically, microbiota-derived short chain fatty acid (SCFA) have been suggested to enhance hematopoiesis in the bone marrow (Trompette et al., 2014). But, commensal intestinal bacteria and products can also affect the development of myeloid cells after haematopoiesis. It has been reported that microbiota-derived SCFA modulates the function of macrophages in the LP due to the inhibition of histone deacetylases (Chang et al., 2014). The continuous replenishment of macrophages in the LP by monocytes expressing C-C chemokine receptor type 2 (CCR2) is also dependent on commensal gut bacteria (Bain et al., 2014). The microbiota has not only a pivotal role in myelopoiesis, but also in regulation of ILCs. Differently to myeloid cells, the development of ILCs is dispensable of the microbiota, though intestinal bacteria are needed for proper functioning of ILCs (Abt et al., 2012; Ganal et al., 2012). For example, tryptophan metabolism by intestinal commensal bacteria (e.g. *Lactobacillus reuteri*) leads to maturation of ILCs (Kiss et al., 2011; Lee et al., 2011; Perdew and Babbs, 1991).

However, not only the postnatal microbial colonization is crucial for immune cell development, but already the maternal microbiota influences innate immune development in the offspring. It has been shown that molecular metabolites of the maternal microbiota impact the development of ILCs and $F4/80^{+}CD11c^{+}$ mononuclear cells *in utero* (Gomez de Agüero et al., 2016).

Bacteria of the microbiota promote also the epithelial barrier function in the intestine. Epithelial cells use microbiota-derived SCFA as energy source, which depletes intracellular oxygen through respiration leading to stabilization of hypoxia-inducible factor (HIF), a transcription factor important for coordinating tissue barrier function (Kelly et al., 2015). Germ-free mice as well as antibiotic treated mice showed reduced luminal SCFA levels and therefore, exhibit diminished HIF, which is connected to a reduced epithelial barrier function (Kelly et al., 2015).

Taken together, the intestinal microbiota greatly affects myeloid and lymphoid cells as well as epithelial cells, which all are crucial for a proper signaling of innate immune cells. Alteration of the microbiota can lead to severe defects in the development and functionality of the innate immune system.

1.2.2 Impact of the microbiota on the adaptive immune system

Intestinal bacteria have a crucial role in the differentiation of cell types of the adaptive immune system, i.e. influencing the functional diversity and repertoire of T and B cells. This is apparent in germ-free mice that develop an immature mucosal adaptive immune system as compared to conventionally raised mice, characterized by with fewer germinal centers in PPs as well as reduced level of IgA-producing B cells and $CD4^+$ T cells (Macpherson and Harris, 2004). In the last decade, researchers provided new mechanistic insights into which specific intestinal members participate in the stimulation of particular adaptive immune pathways.

Several studies have identified an influence of intestinal commensals to direct the differentiation of naïve $CD4^+$ T cells, e.g. to T_H17 cells or T_{reg} cells. T_H17 cells are a subclass of T helper cells, which are characterized by the secretion of the effector cytokine IL-17. T_H17 cells have critical functions in host defense against bacterial and fungal infections, but also can enhance autoimmune and inflammatory diseases (Bettelli et al., 2007). For example, patients with inflammatory bowl disease (IBD) display increases in T_H17 cells and related cytokines in affected parts of the intestine (Maloy and Powrie, 2011; Schmechel et al., 2008). Moreover, together with IL-22 (also secreted by T_H17 cells) IL-17 affects the production of antimicrobial peptides (AMPs) by Paneth cells (Ivanov et al., 2009). Germ-free mice show reduced levels of T_H17 cells in the LP but their numbers are restored after colonization with a complex microbiota (Ivanov et al., 2008). Specifically, a particular commensal, segmented filamentous bacteria (SFB), strongly induces T_H17 cells, which in turn recognize SFB antigens through their T cell receptor (TCR) (Goto et al., 2014b). However, SFB antigens cannot induce T_H17 cell differentiation by itself but is rather induced through physical interaction of SFB adhering to epithelial cells (Atarashi et al., 2015). Moreover, SFB adhesion elicits a

distinct gene expression program in epithelial cells such as upregulation of serum amyloid A, which supports T cell differentiation together with IL-22 (produced by ILC3) (Sano et al., 2015).

Besides the influence of the gut microbiota on pro-inflammatory T_H17 cells, several studies dissected how intestinal members affect the functional development of intestinal $FoxP3^+$ T_{reg} cells. T_{reg} cells exert an immunosuppressive effect by preventing effector T cell induction and proliferation (Harrison and Powrie, 2013). The intestine harbors different types of T_{reg} cells: thymus-derived T_{reg} cells (tT_{reg}) and peripherally derived T_{reg} cells (pT_{reg}). Whereas tT_{reg} cells migrate in the intestine and endure functional maturation, pT_{reg} cell differentiation occurs in the intestine of uncommitted naïve $CD4^+$ T cells (Shevach and Thornton, 2014). pT_{reg} cells are mainly found in colonic LP and express the retinoic acid receptor (RAR)-related orphan receptor γt (RORγt), whereas tT_{reg} cells can be distinguished by its expression of globin transcription factor (GATA) 3 (Tanoue et al., 2016).

Microbial antigens together with TCR signaling initiate T_{reg} differentiation, which is important to inhibit aberrant immune responses to self as well as environmental signals and is therefore, pivotal for intestinal homeostasis (Sefik et al., 2015; Yang et al., 2016). Especially, IL-10 derived from T_{reg} cells, the main producers of IL-10, suppresses the functionality of T_H17 cells (Huber et al., 2011; Rubtsov et al., 2008). Recent studies shed light on how specific bacteria and bacterial derived molecules impact the differentiation and expansion of T_{reg} cells. For example Clostridia of the clusters IV, XIVa and XVIII contribute to accumulation of colonic T_{reg} cells, in particular those which express RORγt, likely through the production of SCFA from dietary fiber (Atarashi et al., 2013; 2011; Sefik et al., 2015). In another study, *Faecalibacterium prausnitzii*, a *Clostridium* specie in the human gut, has been reported to greatly induces IL-10 producing T cells (CD4 and CD8αα cells) and is less abundant in patients with IBD (Sarrabayrouse et al., 2014). Besides bacteria of the class Clostridia, also several bacteria of the class Bacteroidetes, especially of the genus Bacteroides (e.g. *B. fragilis* and *B. thetaiotamicron*) have been demonstrated to affect the induction of intestinal pT_{reg} cells (Faith et al., 2014; Round and Mazmanian, 2010).

Secretory IgA is constantly produced and binds to intestinal bacteria and dietary antigens; therefore, strongly contributes to intestinal homeostasis (Mantis et al., 2011). IgA production is highly dependent on the gut microbiota. Germ-free mice display reduced levels of IgA expressing cells, which is reversed after colonization with gut bacteria (Hapfelmeier et al., 2010; Peterson et al., 2007). In addition, the complexity of the gut microbiota is highly dependent on the diversity of IgA expressing cells (Lindner et al., 2015).

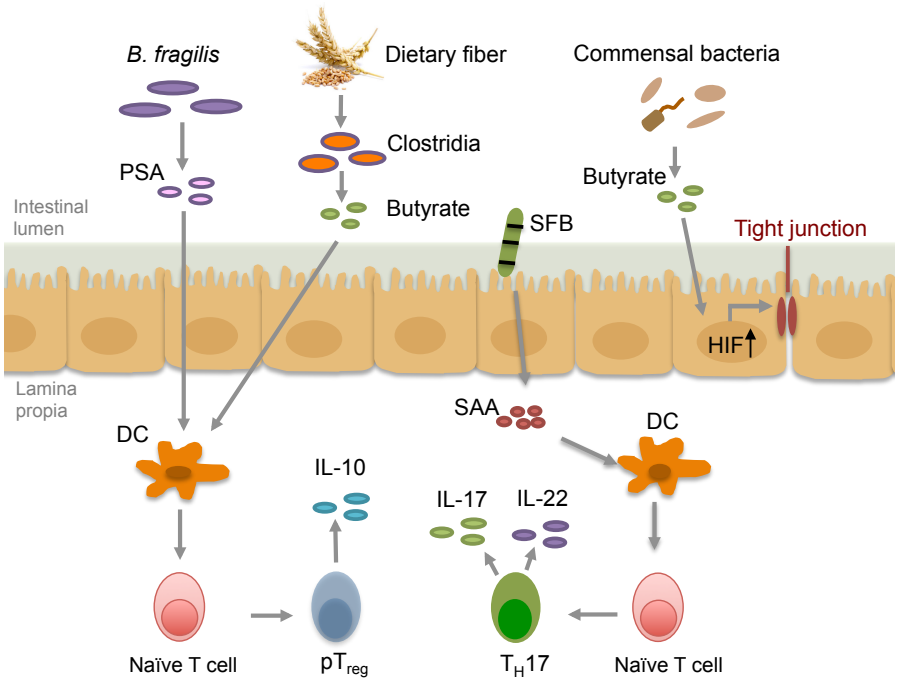

Figure 1.3 - Interactions of commensal bacteria with the mucosal immune system. Intestinal bacteria have a large effect on mucosal immune cells. For example, *Bacteroides fragilis* produces PSA, which in turn promotes the induction of mucosal pT_{reg} cells. In addition, species of the class Clostridia contribute also to pT_{reg} cell induction via production of butyrate from dietary fibre. pT_{reg} cell are an important source of IL-10 controlling intestinal homeostasis. In contrast, SFB promote the development of T_H17 cells via the production of SAA. Moreover, microbiota-derived butyrate is used as energy source by epithelial cells, which in turn, contribute to epithelial barrier function through HIF. DC – dendritic cell, HIF - hypoxia-inducible factor, IL – interleukin, PSA – polysaccharide A, pT_{reg} – peripherally-derived regulatory T cell, SAA – serum amyloid A, T_H – T helper cell, tT_{reg} – thymus-derived regulatory T cell

Taken together, the intestinal microbiota contributes to the functioning of innate immune cells, but also to the development and maturation of the adaptive immune system (Figure 1.3). In the last years, studies have started to uncover how specific members affect distinct cells of the adaptive immune system, including T_H17, T_{reg} and B cells. However, for most of the commensal

gut bacteria it is still unknown how they interact with the mucosal adaptive immune system, likely even also inducing other adaptive immune signaling pathways as shown above.

1.2 The mucosal immune system control the microbiota composition

Not only the microbiota contributes to the functionality of the mucosal immune system, but also innate and adaptive immune pathways have a great effect on the microbial composition in the intestine.

PRRs have an important role in shaping the composition of the gut microbiota and contribute to intestinal homeostasis. Especially in the last years, the impact of PRR signaling on the microbiota was manifested through studies of inflammatory bowel disease, which is characterized by an overactive immune system and diminished epithelial barrier function. Polymorphisms in nucleotide-binding oligomerization domain-containing protein 2 (NOD2), a well-studied member of the NLR family, is associated with increased susceptibly to Crohn's disease (Heresbach et al., 2004). NOD2 recognizes gram-positive and gram-negative bacteria via muramyl dipeptide, constituent of bacterial cell walls. In turn, NOD2 regulates via activation of Rip2 and NFκB the expression of α-defensins by Paneth cells and therefore controls the commensal bacterial microbiota (Lala et al., 2003; Wehkamp et al., 2004). Mice deficient in NOD2 shows an aberrant bacterial community in the terminal ileum characterized by a higher abundance of *Bacteroides* spp. and *Firmicutes* spp. compared to wild type mice (Petnicki-Ocwieja et al., 2009). Another study showed that NOD2 prevents the growth of the commensal gut bacterium *Bacteroides vulgatus* and therefore, controlling intestinal inflammation (Ramanan et al., 2014). Strikingly, two other studies could not reveal any differences in the microbial composition of NOD2-deficient mice and wild type mice (Robertson et al., 2013; Shanahan et al., 2014).

Deficiency in the NLR family pyrin domain containing 6 (NLRP6), mainly expressed in intestinal epithelial cells, leads to alterations of the microbiota composition as well as their function and therefore, increased susceptibility to enteric infections (Elinav et al., 2011; Levy et al., 2015; Wlodarska et al.,

2014). Signaling via myeloid differentiation primary response gene 88 (MyD88), an adaptor protein in TLR signaling, results in enhanced production of AMPs by Paneth cells such as RegIIIγ, a C-type lectin that targets Gram-positive bacteria (Brandl et al., 2007; Frantz et al., 2012). In addition, MyD88 signaling regulates mucin production and therefore, maintaining epithelial barrier function (Bhinder et al., 2014). Loss of MyD88 in epithelial cells enables translocation of opportunistic pathogens such as *Klebsiella pneumonia* (member of the resident gut microbiota) to draining lymph nodes (Frantz et al., 2012). Moreover, loss of MyD88 leads to decreased bacterial abundances of the phylum Bacteroidetes and increased bacterial numbers of the phylum Proteobacteria (Frantz et al., 2012).

In addition, mice deficient in TLR5, a bacterial flagellin receptor, show changes in the composition of the gut microbiota, which was associated with the development of metabolic syndrome including hypertension, insulin resistance and increased adiposity (Vijay-Kumar et al., 2010). Furthermore, transferring gut bacteria of TLR5-deficient mice to wild type germ-free mice has been reported to result in a similar metabolic syndrome as observed in *Tlr5*$^{-/-}$ mice (Vijay-Kumar et al., 2010). TLR5-deficient mice also revealed reduced levels of flagellin-specific IgA, which leads to an upregulated expression of flagella-related genes by several commensal bacteria (Cullender et al., 2013). Similar to what was observed in NOD2 deficient mice, some research groups could not observe alteration in the microbiota in TLR5 knockout mice as seen in the study of Vijay-Kumar et al. (Vijay-Kumar et al., 2010).

Moreover, the mucosal adaptive immune system also contributes to the composition of the microbiota. Humans carrying selective IgA deficiency display a greater abundance of *E. coli* with potentially inflammatogenic properties, which may contribute to the development of gastrointestinal diseases (Friman et al., 2002).

The mucosal host immune system controls the intestinal microbiota via activation of specific innate and adaptive immune responses providing intestinal homeostasis. Defects in the immune system potentially lead to a disrupted intestinal homeostasis and therefore, intestinal dysbiosis, which increases the risk for gastrointestinal diseases and metabolic dysfunctions. As

discussed for NOD2 and TLR5 deficiencies, there is still a debate in the research field which parts of the mucosal immune system regulate the composition of the microbiota. Most of the studies were performed using conventionally raised mouse lines from different vendors, which differ in their microbial composition (Ericsson et al., 2015). Therefore, it is likely that the effect of signaling immune pathways depends also on the resident microbiota composition.

In summary, the interplay between the host and the microbiota drives intestinal homeostasis. Commensal intestinal bacteria have a profound effect on the mucosal immune system, whereas mucosal immune signaling impacts the function as well the composition of the microbiota. Alterations in the composition of the microbiota can lead to profound consequences for the host's physiology, as reported in IBD patients and obesity (Bervoets et al., 2013; Hold et al., 2014). Only a few bacteria are already identified, which regulate inflammatory responses, contributing to host's health and more research is needed in this field. Moreover, a more profound knowledge how bacteria influence the mucosal immune system and contributing to a balanced homeostasis in the gut is crucial to treat microbiota-associated diseases.

1.3 Gastrointestinal infections

Gastrointestinal infections result in inflammation of the gastrointestinal tract causing symptoms such as diarrhea and abdominal pain that lead to death in severe cases. Gastrointestinal infections are transmitted by contaminated food and water as well as contact with infected persons as a result of poor hygiene. According to the World Health Organization, every year 1.7 billion cases of diarrheal disease occur, causing nearly 1.5 million deaths (second leading cause of death in children >5 years old) (WHO, 2013).

A wide range of pathogens, including viruses, bacteria, parasites and fungus, causes gastrointestinal infections. Norovirus, *Campylobacter* spp., nontyphoidal *Salmonella* spp. and pathogenic *Escherichia coli* (shiga toxin-producing *E.coli*) are responsible for the majority of gastroenteritis infection worldwide and are mainly spread by contaminated food (Fletcher et al., 2013). Every year approximately 11.000 cases of Salmonellosis (caused by

Salmonella spp.) are identified (RKI, 2016). Moreover, in 2011, Germany suffered from one of the largest outbreaks of a food-born infection; caused by enterohaemorrhagic *Escherichia coli* (EHEC), a shiga-toxin producing *E.coli* with the serotype O104:H4. Approximately 4.000 patients with EHEC were observed, resulting in 54 deaths (Frank et al., 2011). About 20% of the patients developed a haemolytic uraemic syndrome (HUS), which is characterized by thrombocytopenia (low amount of platelets), microangiopathic hemolytic anemia and kidney failure (Frank et al., 2011).

The development of multiple antibiotic resistances in enteric pathogens is an increasingly global risk problem, in particular in health care units. Therefore, antibiotic resistances threaten the ability to treat common infectious enteric disease. Over the years, the inappropriate antibiotic overuse in humans and animals accelerated the development of multiple antibiotic resistances in bacteria. Moreover, multidrug resistant bacteria can spread easily via the food chain or through the environment (e.g. water and soil) to humans. However, the use of antibiotics is still needed, since it is the most effective treatment against pathogens in human and animals. Therefore, new therapies are needed to combat enteric pathogens, especially for those who acquired antimicrobial resistances and subsequently antibiotic treatment normally fails. For example, exchanging the microbiota of diseased patients by stool transplantation from healthy persons was reported to eradicate antibiotic-resistant pathogens (Millan et al., 2016). Thus, it is important to understand in detail, how pathogens interact with the host exploiting their virulence factors for colonization and which specific role the microbiota has to confer resistance to enteric infections. In the next part, I will focus on two enteric pathogens, *Salmonella enterica serovar* Typhimurium and *Citrobacter rodentium*, which are both used in this thesis to identify microbial signatures associated with lower disease severtiy (Chapter 2 and 3). Moreover, I will explain how commensal bacteria combat enteric infections.

1.4.1 *Salmonella enterica* ssp. enterica serovar Typhimurium

Salmonella spp. are a group of gram-negative food-borne pathogens belonging to the bacterial family of Enterobacteriaceae. The group of

Salmonella comprises two species (*S. enterica* and *S. bongori*) and six subspecies, including more than 2500 serovars (Coburn et al., 2007). *S. enterica* can infect a broad range of species causing gastroenteritis and typhoid fever, depending on the serovars. For example, *S.* Typhi disseminate systemically by spreading throughout the peripheral lymphatic system, causing typhoid fever (Everest et al., 2001). In contrast, most of the serovars of *S. enterica* cause nontyphoidal symptoms, triggering rapid inflammatory responses, causing an estimated 93.8 million cases worldwide per year (Majowicz et al., 2010).

In contrast to *S.* Typhi, the nontyphoidal *Salmonella enterica* serovar Typhimurium can not only infect humans but also induce gastroenteritis in other mammalians. Therefore, *S.* Typhimurium is also used in mouse models to study immune responses to *Salmonella* and bacterial virulence factors. In mice, *S.* Typhimurium can colonize the gastrointestinal tract and triggering a strong gut inflammation, but can also spread via the GALT to colonizes systemic sites (Carter and Collins, 1974). Infection with *S.* Typhimurium in normal laboratory mice results in a poor luminal colonization. *S.* Typhimurium disseminate in systemic organs similar to what is known in human typhoid diseases (e.g. infection with *S.* typhi) and therefore, is used as a mouse model to study human typhoidal *Salmonella* infections (Tsolis et al., 1999). In contrast, antibiotic treatment in mice before *S.* Typhimurium infection results in strong gastroenteritis, showing similar characteristics as seen in human nontyphoidal infections. Therefore, the antibiotic-induced (e.g. streptomycin) *S.* Typhimurium model is a well-established mouse model to study *Salmonella*-induced gastrointestinal inflammation (Barthel et al., 2003).

S. Typhimurium elicits mucosal inflammation by utilizing virulence factors and triggering inflammation by PRRs. To invade the tissue and to cause gastroenteritis, first *S.* Typhimurium closely binds to enterocytes by expression of adhesins and its flagellin (Bäumler et al., 1996; Stecher et al., 2008). S. Typhimurium elicits mainly gut inflammation via two type III secretion systems (T3SS), T3SS-1 and T3SS-2, which are encoded in the *Salmonella* pathogenicity island (SPI)-1 and SPI-2, respectively. Both T3SSs trigger inflammation independently of each other. T3SS-1 induces

inflammation within the first 6-10 hours post infection (p.i.) and T3SS-2 triggers enteropathy at 3 days p.i. (Kaiser et al., 2012).

Via the T3SS-1, *S.* Typhimurium delivers effector proteins such as SipA and SopE into the host cell, promoting pathogen entry into epithelial cells and triggering different pro-inflammation responses (Müller et al., 2009; Wall et al., 2007). For instance, SipA facilitates bacterial invasion by binding to actin and inhibiting actin depolymerization (Zhou et al., 1999). SipA also induce the activation of epithelial pathways leading to neutrophil migration (Lee et al., 2000). It has been shown that SipA mediates NOD1/NOD2 signaling that activates NF-κB-dependent inflammatory responses (Keestra et al., 2011). Moreover, NOD1/NOD2 signaling was identified to trigger early IL-17 production (Geddes et al., 2011).

SopE, a guanine nucleotide exchange factor, activates host cellular Rho GTPases. For example the two Rho GTPases Rac1 and Cdc42 support cytoskeletal rearrangement and therefore, help to engulf *Salmonella* (Hardt et al., 1998). Activation of Rac1 and Cdc42 induce also NOD1 signaling and consequently, activates RIP2-NF-κB-dependent host inflammatory responses (Keestra et al., 2013). Moreover, SopE supports caspase-1 activation and their cytokines IL-1β and IL-18 to trigger mucosal inflammation (Müller et al., 2009).

In contrast to T3SS-1, T3SS-2 is activated after *Salmonella* host cell entry. Colonization of the LP via the T3SS-2-dependent pathway (in absence of T3SS-1) is mediated by $CD11c^{+}CX3CR1^{+}$ monocytic phagocytes (Hapfelmeier et al., 2008). T3SS-2 enables *Salmonella* to persist and replicate within a cellular vacuolar department of macrophages and dendritic cells. In turn, different PRRs are activated such as TLRs to mediate inflammatory responses. For instance, TLR2 and TLR4 and their downstream adaptor molecule Myd88 are pivotal for host defense against *Salmonella* infection (Weiss et al., 2004).

As discussed above, upon infection and stimulation of different PRRs, innate and adapted mucosal immune responses are initiated (Figure 1.4). The induction of inflammatory cytokines, including IL-1β, IL-18 and IL-6, help the host to combat infection. Another important induced cytokine is interferon (IFN)γ, which is mainly produced by natural killer cells (NK) and T cells,

supporting lowering pathogen loads in the mucosal tissue (Kupz et al., 2013; Srinivasan et al., 2004).

Besides the inflammatory response of the host to constrain *Salmonella* infection, also the host microbiota is an important factor to control pathogen loads. In conventionally raised mice, *S.* Typhimurium establishes a poor luminal colonization (Barthel et al., 2003). This phenomenon is referred to as colonization resistance, which explains interactions of the microbiota with the pathogen and host to protect against invading pathogens (van der Waaij et al., 1971). Germ-free mice and antibiotic treatment prior *S.* Typhimurium infection in mice (e.g. treatment with a single dose of streptomycin, ampicillin or vancomycin) leads to high pathogen colonization in the lumen and gut tissue, resulting in severe intestinal inflammation already after early time points of infection (Barthel et al., 2003; Stecher et al., 2005). Therefore, the microbiota has a large effect on disease progression and disruption of the microbiota enhances disease susceptibility.

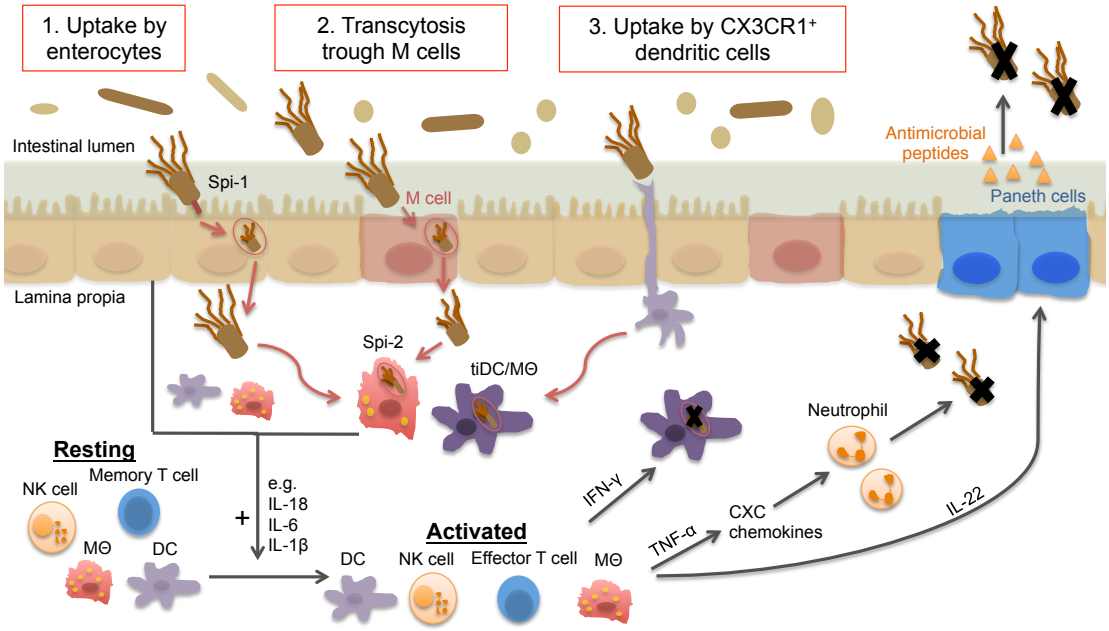

Figure 1.4 – *Salmonella* invasion and host immune response. *Salmonella* invades the host via different routes. (1) *Salmonella* invade enterocytes through the injection of effector proteins, which is mediated by SPI-1 Type 3 secretion system. (2) M cells transport *Salmonella* to the lamina propria by transcytosis. (3) CX3CR1^{+} DCs take up *Salmonella* with their transepithelial dendrites. After detection of *Salmonella* by PRRs, an immune response is initiated, which leads to production of pro-inflammatory cytokines (e.g. IL-6, IL-1β). In turn, adaptive immune cells are activated, targeting intra- and extracellular *Salmonella*. Influx of neutrophils leads not only to killing of pathogens, but also damage epithelium, causing loss of epithelial barrier function. IFN – interferone, IL – interleukin, M cell – microfold cell, MΘ – macrophages, NK cell – natural killer cell, Spi - *Salmonella* pathogenicity island, tiDC – tissue residing dendritic cell, TNF – tumor necrosis factor

However, *Salmonella* also developed mechanisms to exploit the microbiota and host mucosal defense for its own outgrowth and colonization. For instance, during inflammation the host produces large amounts of lipocalin-2, which sequester the siderophore enterochelin in the gut lumen. Siderophores are needed for the efficient uptake of iron. For example*, E. coli* produces enterochelin that is pivotal to import iron via the *fep* ABC transporter (Buchanan et al., 1999). In case of *S.* Typhimurium, it produces salmochelin, which is not bound by lipocalin-2 and therefore, *Salmonella* can still use iron during gut inflammation giving it a growth advantage towards commensal bacteria as *E. coli*.

To summarize, *S.* Typhimurium uses its virulence factors to cross the epithelial barrier colonizing the LP and to persist in host immune cells. On the other side, the host activates the innate and adaptive mucosal immune system to combat against infection. Intriguingly, *S.* Typhimurium also benefits from the host inflammatory response. However, it has been shown that the community of intestinal commensal bacteria greatly influences disease progression. But which specific members of the microbiota affect *Salmonella* colonization are still not completely identified. Moreover, is not clear yet, how these specific members interact with *Salmonella* or the host to prevent pathogen colonization.

1.4.2 *Citrobacter rodentium*

Citrobacter rodentium is a mouse-restricted pathogen, which is used as model for the human pathogens enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E.coli* (EPEC). Similar to EHEC and EPEC in humans, *C. rodentium* is a highly infectious pathogen causing colitis and colonic hyperplasia in mice (Luperchio et al., 2000). *C. rodentium* is a member of the family Enterobacteriaceae and genetically related to EPEC and EHEC, sharing about 67% of their genes, including important homolog virulence factors (Luperchio et al., 2000; Petty et al., 2010). Moreover, *C. rodentium* is transmitted by the fecal-oral route and colonize the gut mucosa by formation of attaching/effacing (A/E) lesions on enterocytes, which are indistinguishable from those triggered by EPEC and EHEC (Schauer and Falkow, 1993). A/E

lesions are formed by attachment to intestinal epithelial cells, effacement of the brush border villi and formation pedestal-like extensions of the epithelial cells beneath the adherent bacteria (Schauer and Falkow, 1993). To induce A/E lesions, *C. rodentium* expresses the adhesion molecule intimin, T3SS and effector proteins, which are encoded on the locus of enterocyte effacement (LEE) pathogenicity island, also shared by the pathogens EPEC and EHEC (Deng et al., 2001). In the host cell translocated effector proteins (e.g. Tir, Esp, Nls) trigger signal transduction and actin polymerization, which is important to form A/E lesions (Deng et al., 2003; Kelly et al., 2006; Mundy et al., 2004). In addition to the T3SS, *C. rodentium* also express a type IV pilus, which is important for colonization possibly due to its role in adherence of epithelial cells as well as two type VI secretion systems (CTS1 and CTS2), which may contribute to colonization resistance by outcompeting intestinal bacteria (Gueguen and Cascales, 2013; Mundy et al., 2003; Petty et al., 2010).

C. rodentium induces a variety of host defense mechanisms upon being recognized by different PRRs. For instance, deficiency in Myd88 leads to increased colitis and higher luminal bacterial numbers (Gibson et al., 2008). Moreover, several NLRs have been identified to be activated upon *C. rodentium* colonization and helping the host to combat the infection. Intracellular NOD2 sensor regulated clearance of *C. rodentium* by CCL2-dependent recruitment of inflammatory monocytes and IL-12 production (Kim et al., 2011). Additionally, mice lacking components of the NLRP3-Caspase-1-IL-1β pathway results in higher bacteria burden, indicating that this pro-inflammatory axis is critical for host defense against *C. rodentium* (Liu et al., 2012). As already mentioned before, activation of PRRs increases the production of pro-inflammatory cytokines to combat *C. rodentium* infections. Besides the aforementioned cytokines IL-22, IL-1β and IL-18, production of IL-17 during infection plays a crucial role in intestinal pathology. Early IL-17 production of $CD4^+$ T cells was dependent on NOD1 and NOD2 sensors, controlling mucosal immunity against *C. rodentium* (Geddes et al., 2011; Torchinsky et al., 2009).

Similar as discussed in *S.* Typhimurium infections, the microbiota has a great impact to control *C. rodentium* colonization and disease progression. It has

been demonstrated, that germ-free mice showed a higher luminal pathogen load (Kamada et al., 2012). The same study showed that the microbiota is needed for eradication of *C. rodentium* (Kamada et al., 2012). Moreover, alteration of the microbiota composition due to antibiotic treatment (e.g. with metronidazole) increases *C. rodentium* colonization (Wlodarska et al., 2011). These studies indicated the importance of the microbiota for disease pathogenicity.

Altogether, *C. rodentium* is a good model to study A/E pathogens including their colonization and virulence mechanisms as well as the activation of innate and adaptive mucosal immune pathways. However, the microbiota has been described to affect different parts of the disease progression. But how single bacteria of the gut microbiota can influence disease outcome is completely not understood.

1.4 Impact of the microbiota on pathogen colonization

In the gastrointestinal tract, commensal bacteria are crucial for protecting the host against invading pathogens. To confer resistance, commensal bacteria developed different mechanisms to inhibit harmful pathogens. Nevertheless, commensal bacteria and pathogen share the same environment such as nutrients and niches. Therefore, the pathogen also evolved mechanisms to exploit microbiota-derived products.

1.5.1 Microbiota-mediated colonization resistance

One of the most important functions of the microbiota is to support resistance to colonization of exogenous pathogens in a process referred to as colonization resistance. Already five decades ago, Bohnhoff and Miller identified that mice exhibit a higher susceptibility to *S.* Typhimurium when treated with antibiotics and pointed out the clinical problem of infections in patients undergoing antibiotic treatment (Bohnhoff and Miller, 1962). Since then other researcher also highlighted the importance of the gut microbiota by demonstrating that germ-free mice as well as antibiotic treated mice with an altered microbiota composition and reduced diversity display a higher

susceptibility to several enteric pathogens (Bohnhoff and Miller, 1962; Kamada et al., 2012; Ng et al., 2014; Sprinz et al., 1961).

Researchers could identify several mechanisms how commensal bacteria combat against pathogen colonization. The gut microbiota confers pathogen colonization resistance via different ways of defense mechanisms: direct inhibition, activation of the immune system and maintenance of the epithelial barrier (McKenney and Pamer, 2015) (Figure 1.5).

Intestinal bacteria affect pathogen growth by competing for nutrients or by activating inhibitory molecules. For instance, bacteria can produce AMP such as bacterocin. *Lactobacillus lactis* produces the bacterocin nisin, which was shown to target the growth of *C. difficile* (Le Lay et al., 2016). Also the two-component bacterocin thuricin CD produced by *Bacillus thuringiensis* shows activity against *C. difficile* (Rea et al., 2010). Moreover, secondary bile acids, which are generated by commensal bacteria from primary bile acids have been shown to inhibit the growth of *C. difficile* (Buffie et al., 2015). In addition, the consumption of specific nutrients by commensal bacteria contributes to competitive exclusion of the pathogen by limiting nutrients availability. For instance, commensal *E. coli* strains inhibit growth of pathogenic *E. coli* because of similar nutrient utilization (Leatham et al., 2009). *B. thetaiotaomicron* has the ability to inhibit *C. rodentium* infection by consuming structurally similar carbohydrates (Kamada et al., 2012).

In contrast to direct mechanisms, indirect ones require microbiota dependent activation of the immune system, which in turn, targets pathogen colonization by e.g. the production of AMPs. Polysaccharide A (PSA) from the capsule of the human commensal *Bacteroides fragilis* induces the conversion of $CD4^+$ T cells into $Foxp3^+$ T_{reg} cells, which in turn, enhances the IL-10 production protecting mice from *Helicobacter hepaticus* induced colitis (Round and Mazmanian, 2010). The mouse commensal bacteria SFB mediates T_H17 activation resulting in IL-22 and IL-17 production, which induces the release of AMPs targeting *C. rodentium* (Goto et al., 2014b; Ivanov et al., 2009; Yang et al., 2014). Also commensal bacteria influence *S.* Typhimurium infection. It was shown that gut bacteria activate Myd88- and NOD2-dependent pathways to express several AMPs, which inhibit colonization of *S.* Typhimurium (Ayabe et al., 2000; Vaishnava et al., 2008).

The gut microbiota contributes also to maintenance of the intestinal barrier, which influences strongly the tissue colonization of pathogenic bacteria. Mucin is produced and secreted by goblet cells, which are simple columnar epithelial cells, scattered among the gastrointestinal tract. Gut bacteria are crucial for proper mucus development. Germ-free mice display a thinner mucus layer and by bacteria more penetrable, which is reverted by restoring a normal microbiota (Johansson et al., 2015). Moreover, the bacterial outer membrane component lipopolysaccharides (LPS) promotes the thickness of the mucus layer (Petersson et al., 2011). Antibiotic treatment disrupts the mucosal barrier supporting bacterial invasion (Wlodarska et al., 2011).

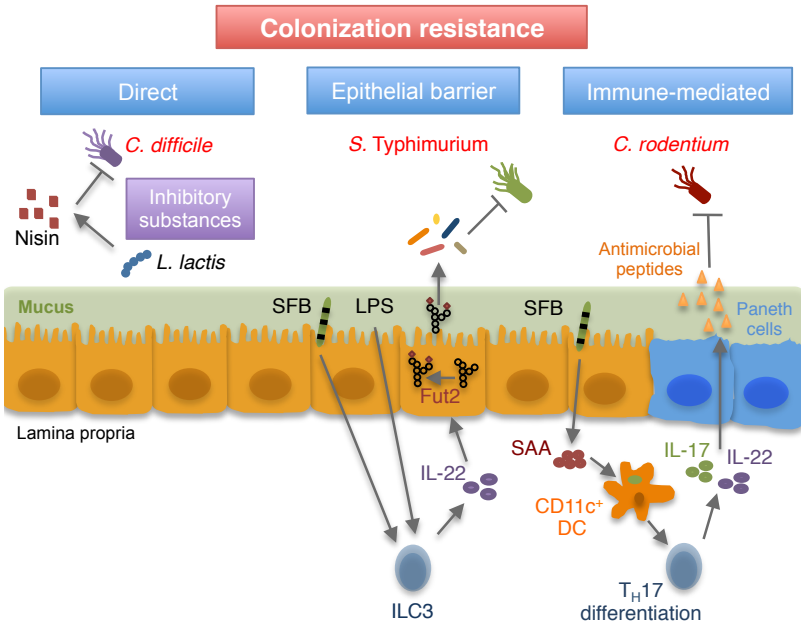

Figure 1.5 - Mechanisms of colonization resistance. Commensal bacteria support the host against invading pathogens through different mechanisms. For example, direct mechanisms include the competing for nutrients, inhibiting pathogen growth. In addition, commensal bacteria increase epithelial barrier function. For example, production of IL-22 induced by commensal bacteria leads to induction of Fut2 expression, contriburing to bacterial diversity, which decreases pathogen colonization. Furthermore, commensal bacteria confer resistance by activating the immune system, which in turn target the pathogen. For example, SFB enhances antimicrobial peptide expression, inhibiting *Citrobacter rodentium* growth. DC – dendritic cell, Fut2 – Fucosyltransferase 2, IL- interleukin, ILC – Innate lymphoid cells, LPS – Lipopolysaccharides, SFB – Segmented filamentous bacteria, SAA – Serum amyloid A, T_H – T helper cell (Adapted from Thiemann et al., 2016)

Moreover, the enzyme 2-α-L-fucosyltransferase 2 (FUT2) adds fucose residues to intestinal mucins (Hurd et al., 2005). Fucose is used as substrate of commensal intestinal bacteria supporting the bacterial diversity and in turn, decrease pathogen invasion (Pickard et al., 2014). The mucus fucosylation

status is microbiota-dependent via the induction of IL-22 by type 3 innate lymphoid cells (ILCs) (Goto et al., 2014a). Mice deficient in *Fut2* show a higher bacterial burden of *S.* Typhimurium and *C. rodentium* (Goto et al., 2014a; Pickard et al., 2014). Moreover, administration of fucosylated molecules to *C. rodentium* infected *Il22ra1*$^{-/-}$ mice helps to restore diversity of the gut community, which influences enteric infection (Pham et al., 2014).

Altogether, microbiota-dependent host protection is mediated by direct mechanisms, immune system induced indirect pathways and by the maintenance of the epithelial barrier. However, the microbiota harbors a diverse community of bacteria, which protect the host via different, specific mechanisms. Until now, researches could identify only a few mechanisms how single bacteria can limit pathogen colonization. Finding new mechanisms are crucial to develop mucosal therapies to combat against gastrointestinal diseases.

1.5.2 Pathogen exploitation of the microbiota

The microbiota protect the host for pathogen colonization. To overcome the microbiota, the pathogen evolved mechanisms to invade the host by exploiting the microbiota. The gastrointestinal tract contains a taxonomical diverse bacterial ecosystem, generating a wide variety of metabolites. Enteric pathogens use those microbiota-derived products such as sugars, organic and inorganic compounds as nutrients to colonize and deploy their virulence genes.

Distinct intestinal commensals, mainly members of the phylum Bacteroidetes, are able to degrade complex food- and host-derived carbohydrates due to their expression of specific hydrolases. These microbiota-liberated sugars are an important nutrient source for enteric pathogens, which lack enzymes for that degradation. For instance, *S.* Typhimurium and *C. difficile* catabolize sialic acid, one of the components of the mucus layer. Since both pathogens cannot liberate the sugar out of the mucus layer, they require sialidase activity of commensal bacteria (Ng et al., 2014). Colonization of germ-free mice with the sialidase-deficient intestinal commensal, *B. thetaiotaomicron*, decreases the level of liberated sialic acids, thereby reducing the colonization of *C.*

difficile (Ng et al., 2014). *B. thetaiotaomicron* not only degrade sialic acid from the mucus, it also liberates fucose from host glycans by producing fucosidase. In turn, EHEC senses free fucose, which enhances their colonization by increasing expression of virulence genes (Pacheco et al., 2012).

The microbiota produces also distinct by-products, derived from anaerobic fermentation, which enteric pathogens can use for growth advantage. One example is the microbiota-derived byproduct hydrogen sulfide (H_2S). During gastrointestinal inflammation, enterocytes transform H_2S to tetrathionate, in a neutrophil- and reactive oxygen species (ROS)-dependent pathway. In turn, *S.* Typhimurium uses tetrathionate as electron acceptor for anaerobic respiration (Winter et al., 2010). Moreover, *S.* Typhimurium utilize microbiota-derived hydrogen as an energy source for outgrowth (Maier et al., 2013). Another microbiota by-product, succinate, is metabolized as carbon source for *C. rodentium* growth enhancing their virulence genes. Levels of succinate is significantly higher in *B. thetaiotaomicron* reconstituted mice (Curtis et al., 2014). Besides succinate, also SCFA induce the expression of different virulence genes in *S.* Typhimurium (Spi-1) and EHEC (LEE PAI) (Lawhon et al., 2002; Takao et al., 2014).

In summary, intestinal bacteria provide access to different sugars, electron acceptors, organic and inorganic compounds, which enteric pathogens exploit as nutrient source or to increase virulence signals. However, the gastrointestinal tract harbors a great diversity of commensal bacteria, forming a complex metabolic network, which is still challenging to study. Therefore, many pathways, how pathogens exploit the microbiota for their advantage, are still not identified.

1.5 Tools for analyzing the gut microbiota

Studying the composition and the functionality of the microbiota is still challenging. Different “omics” approaches, including culture-dependent and culture-independent technologies, are needed to study the composition of the microbiota as well as the interaction with the host, the pathogen and other commensal bacteria.

Until 10 years ago, the characterization of the gut microbiome was driven by culture-based techniques and research was focused on bacteria, which were culturable. But, due to the complex gut ecosystem and its tight regulated interplay between intestinal bacteria and the immune system, numerous intestinal bacteria are difficult to culture. Therefore, culture-based techniques do not represent the whole gut microbiota (Browne et al., 2016; Rappé and Giovannoni, 2003). Hence, culture-independent, DNA-based next-generation sequencing technologies (NGS) offered new opportunities to study the complex ecosystem of the microbiota in the last decade. Both, targeted sequencing as well as whole-genome sequencing technologies are used to study bacterial communities.

Sequencing of targeted marker genes provides a rapid, cost-efficient and precise approach to investigate the diversity and composition of a microbial community. The 16S ribosomal RNA (16S rRNA) gene is the most frequently targeted marker gene and is used for standard identification of microbes due to its ubiquitous presence in prokaryotes. The 16S rRNA gene belongs to the prokaryotic ribosome and contain highly conserved as well as hyper variable regions (V1-V9). Therefore, the 16S rRNA gene provides species-specific signatures, which allows distinguishing different taxa. Moreover, 16S rRNA-based techniques permit to identify uncultivable bacteria.

Highly conserved 16S rRNA regions (slow evolving regions) are used to design PCR primers, which amplify also the highly variable 16S rRNA regions (Kuczynski et al., 2011). The design of those PCR primers is critical for taxonomic coverage and specificity of amplified bacterial sequences, depending on the host species (e.g. mouse, human) and the target bacteria. For instance, targeting the variable region V1-V2 results in under representative taxa as *Bifidobacterium* and Verrucomicrobia (Bergmann et al., 2011; Hayashi et al., 2004), whereas V1-V2 best differentiate among *Staphylococcus* species (Chakravorty et al., 2007). Currently, the variable regions V1-V2 or V4 are most commonly used to study the gut microbiota.

However, 16S rRNA studies do not allow gaining insights into microbial functionality, because only a small fragment of the whole genome is analyzed. Furthermore, annotation of bacteria is based on OTUs with 97% similarity, providing data, which are less accurate at bacterial species and strain level.

Characterizing whole bacterial genomes by metagenomic studies is used to describe the functional potential of represented bacteria. Different than 16S rRNA sequencing, metagenomic approaches such as whole genome shotgun (WGS) sequencing have the advantage to not only targeting a specific gene. Therefore, bacteria can be more precisely determined at species level, if a good database is available (Ranjan et al., 2016). Nevertheless, metagenomic studies have a lower resolution of the present bacteria in the analyzed community as 16S rRNA sequencing (Kuczynski et al., 2011). In addition, compared to 16S rRNA sequencing, metagenomic studies are also a more expensive approach. Moreover, a more complex data analysis is needed.

However, by using DNA based methods such as 16S rRNA sequencing and metagenomic of DNA samples, vitality of bacteria is not assessed. Therefore, RNA-based studies by sequencing 16S rRNA transcripts or whole transcriptomes (metatranscriptomic studies) provide insights into the viable community. Moreover, to couple these data with metaproteomics and metabolomics studies is essential to understand the metabolic activity and therefore functionality of the bacteria in the intestine.

In the last years, especially Illumina-based sequencing platform were used more frequently in laboratories due to its high number of reads and low cost (Weinstock, 2012). Illumina sequencing is based on the incorporation of fluorescently labeled deoxyribonucleotide triphosphates (dNTPs) into a synthesizing DNA template strand. Before DNA sequencing, 5’ and 3’ strand adapters are added to the target gene, which binds in an Illumina flow cell to surface-bound oligos complementary to the adapters and is amplified in clonal clusters through bridge amplification. During DNA synthesis, incorporated nucleotides are identified by fluorophore excitation. Therefore, millions of fragments can be sequenced simultaneously. To process a large number of samples, multiplexing of samples is a useful technique that is accomplished due to adding 5’ and 3’ strand barcode sequences by PCR to distinguish several samples after sequencing. Following sequencing of the target gene, these millions of generated reads needs to be aligned and clustered based on their similarity into operational taxonomic units (OTUs) to assign the DNA sequences to bacterial species. Reads are normally clustered together, which have a 97% similarity.

Culture-independent DNA and RNA-based sequencing technologies are an essential tool to identify microbial communities and provide knowledge about bacteria, which are associated to specific health and disease states. However, culture-dependent techniques coupled with animal models contribute crucially to the urgently need to show causality rather than association and to study microbial interactions between the microbiota and the host.

However, isolation of novel bacterial species is laborious. Most of the intestinal bacteria require anaerobic conditions. Moreover, some bacteria need for their growth the interaction with other microorganism, e.g. to provide essential nutrients. These interactions are often complex and largely unknown. In the last years, more attention has been paid to large-scale isolations and new culture techniques. Those microbial culturomics data include for example the use of different media, temperature, pH and atmospheres, which are coupled with MALDI-TOF mass spectrometry (MS) studies (Lagier et al., 2012). Data of those studies possibly simplify to set up the best conditions to isolate novel bacterial species.

Moreover, animal models allow verification of correlation studies in a controlled experimental setup as well as development of mechanistic and functional research on host-microbe interactions. Especially, microbial interactions with the host immune system are hard to study in human cohorts. Furthermore, our recent insights of the host-microbiota interplay result mainly from studies with germ-free mice. Germ-free mice do not harbor any microorganisms and are raised in isolators to ensure a microorganism-free environment, including bacteria, viruses and eukaryotes (Rooks and Garrett, 2016). A vast number of wild type mouse strains and knockout strains are available in germ-free conditions. With the help of germ-free mice, researchers can inoculate specific bacteria or a defined bacterial mix to generate a desired microbiota. Those mice are referred to as gnotobiotic mice, which also can be further challenged (Rooks and Garrett, 2016). Moreover, mice with a defined humanized microbiota can be generated (Goodman et al., 2011). In the future, these experiments could be extended to mice with a humanized immune system.

In the last decade, genomic-approaches have greatly advanced our understanding about complex microbial ecosystems in a rapid and cost-

efficient way. However, still many of the intestinal bacteria could not yet be isolated. Nevertheless isolation of bacterial species is important to show direct causality and is crucial for development of novel bacterial therapeutics in the future (Walker et al., 2014) .

In summary, a combination of culture-independent and –dependent tools is required to fully understand the complexity of the microbiota and their functionality.

1.6 Strategies to manipulate the gut microbiota

Several diseases are associated with an altered gut microbiota, such as IBD, colorectal cancer as well as obesity and diabetes (Carding et al., 2015). Moreover, studies have associated lower microbial diversity with increased susceptibility to intestinal diseases (Manichanh et al., 2006; Png et al., 2010), which might be traced back to the lack of specific beneficial bacteria. Therefore, a great interest started to manipulate the microbiota in order to confer benefits for host's health. Currently, transfer of complex communities from a healthy donor to a diseased recipient (known as fecal transplantations) as well as the use of novel prebiotics and probiotics are investigated by researchers; especially, for the urgent need to decrease susceptibility to enteric multi-drug resistant infections.

In the last years, fecal transplantation as therapy gained great success due to the amelioration of recurrent *C. difficile* infection (CDI), possibly due to enhancement of diversity and richness of the recipient's microbiota. For instance, CDI was strongly associated with a dysbiotic status of the microbiota, mainly after antibiotic treatment (Stevens et al., 2011). Recurrent fecal transplantation to patients with recurrent CDI showed efficiency rates of about 90% (Landy et al., 2011). Moreover, the still increasing demand for fecal transplantations requires the development of frozen stool banks in analogy of blood blanks, which simplifies the screening for donor samples regarding potential pathogens and recipient's compatibility (Paramsothy et al., 2015). In addition, strict criteria regarding donors BMI, blood counts and inflammatory markers are crucial for selection of fecal transplantation donors.

Nevertheless, fecal transplantation harbors several risk factors. Transferring a bulk community could potentially include opportunistic pathogens, which potentially poses significant regulatory hurdles. Moreover, it is difficult to completely identify the community using NGS based approaches. Even small amounts of harmful bacteria possibly could have tremendous long-term effects on the recipient's host. Additionally, bacteria of the fecal transplant could possibly change their behavior in the new environment of the recipient due to altered host genotype, diet and the residual commensal microbiota. Therefore, beneficial commensals could potentially develop to harmful bacteria.

Because fecal transplantation is associated with several risks, the need for transferring well-characterized bacterial communities is rising. Different studies have already been shown that bacterial mixtures prevent CDI. Already 15 years ago, in a small-scale study Tvede and Rask-Madsen identified a mixture of 10 isolated bacteria, which was effective in patients with CDI (Tvede and Rask-Madsen, 1989). More recently, a study with 33 isolated bacteria could cure CDI in patients, who had failed at least three courses of antibiotic treatment (Petrof et al., 2013).

Overall, fecal transplantation is a promising therapeutic approach, in particular for enteric infections such as CDI. Though, fecal transplantation harbors several risks as transfer of inadvertent harmful bacteria. Therefore, identifying a mixture of beneficial bacteria or a single bacterium (probiotics) as well as the discovery of substances that induce the growth of beneficial bacteria (prebiotics) is needed. Until then, fecal transplantation offers a promising intermediate treatment.

1.7 Aim of the study

The microbiota protects the host against invading pathogens through various mechanisms. However, which distinct members of the microbiota decrease susceptibility as well as how they protect the host is far from being completely understood. The aim of the study is to identify commensal gut bacteria, which are associated with decreased susceptibility to gastrointestinal infections. Isolating of associated bacteria is important to investigate the causality. Moreover, identifying how target bacteria confer colonization resistance (e.g. direct mechanisms, immune-mediated indirect pathways or by the maintenance of the epithelial barrier) is crucial to understand the important interplay between the microbiota and the pathogen. To conduct these studies, mice harboring different microbiota compositions are infected with the murine gastrointestinal pathogens *S.* Typhimurium and *C. rodentium* with the aim to:

1. **Finding isogenic mouse lines with distinct microbiota compositions, which differ in their disease susceptibility to enteric infections:** Isogenic mouse lines with different microbiota compositions are infected with enteric mouse pathogens *S.* Typhimurium and *C. rodentium*. Using cohousing and fecal transplantation from resistant to susceptible mice help to understand if the resistant phenotype can be transferred in before susceptible mice.
2. **Identifying bacterial signatures correlating with decreased susceptibility to enteric infections:** 16S rRNA sequencing at different time points offer valuable information about intestinal bacteria associated with reduced susceptibility against *S.* Typhimurium and *C. rodentium* infections.
3. **Isolating intestinal bacteria conferring resistance:** Isolating bacteria associated with higher resistance is required to verify correlation studies (referred to bullet point 2). Therefore, for isolation aerobic and anaerobic culturing methods are applied. Transferring isolated bacteria in susceptible mice and analyzing the susceptibility to gastrointestinal infections is important to show direct causality.

4. **Characterizing bacterial mechanisms important for resistance:** How intestinal bacteria affect pathogen colonization is analyzed to identify novel mechanisms critical for host protection against gastrointestinal pathogens. Therefore, mice deficient in important immune pathways and with additionally different microbiota compositions are used.

The results of the study contribute to understand in more detail, which intestinal bacteria are important to confer colonization resistance to gastrointestinal pathogens. Moreover, the study provides insights into mechanisms to confer resistance to invading pathogens. Identifying the basic principles of immune regulation by commensals may allow harnessing their abilities to develop novel mucosal therapies for gastrointestinal infections.

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Abbreviations

A/E	Attaching/effacing
AMP	Antimicrobial peptide
CCL	CC-chemokine ligand
CCR	C-C chemokine receptor type
CD	Cluster of differentiation
CDC42	Cell division control protein 42 homolog
CDI	*Clostridium difficile* infection
DC	Dendritic cell
EHEC	Enterohaemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *E.coli*
Esp	Escherichia secreted proteins
FOXP3	Forkhead box protein 3
FUT2	Fucosyltransferase 2
GALT	Gut-associated lymphoid tissue
GATA	Globin transcription factor
H_2S	Hydrogen sulfide
HCS	Hematopoietic stem cell
HIF	Hypoxia-inducible factor
IBD	Inflammatory bowl disease
IEC	Intestinal epithelial cell
IFN	Interferon
IgA	Immunoglobulin A
IL	Interleukin
ILC	Innate lymphoid cell
ILF	Isolated lymphoid follicle
LEE	Locus of enterocyte effacement
LP	Lamina propria
LPS	Lipopolysaccharides
M cells	Microfold cells
MALDI	Matrix-assisted laser desorption/ionization
MAMP	Microbe-associated molecular pattern
MS	Mass spectrometry
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B-cells

NGS	Next generation sequencing
NK cell	Natural killer cell
NLR	NOD-like receptor
NLRP6	NLR family pyrin domain containing 6
Nls	Nuclear localization signal
NOD	Nucleotide-binding oligomerization
NTP	Deoxyribonucleotide triphosphates
OTU	Operational taxonomic units
p.i.	Post infection
PAMP	Pathogen-associated molecular pattern
PP	Peyer's Patches
PRR	Pattern-recognition receptor
PSA	Polysaccharide A
pT_{reg}	Peripherally-derived T_{reg}
Rac1	Ras-related C3 botulinum toxin substrate 1
RAR	Retinoic acid receptor
Ras	Rat sarcoma
Rho	Ras homologues
Rig I	Retinoic acid-inducible gene I
RIP2	Receptor-interacting protein 2
RLR	Rig I-like receptor
RORγt	RAR-related orphan receptor γt
ROS	Reactive oxygen species
SAA	Serum amyloid A
SCFA	Short chain fatty acid
SFB	Segmented filamentous bacteria
SipA	Salmonella invasion protein A
SPI	*Salmonella* pathogenicity island
T3SS	Type III secretion system
TCR	T cell receptor
T_H	T helper lymphocyte
Tir	Translocated intimin receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOF	Time of flight
T_{reg}	Regulatory T cells
tT_{reg}	Thymus-derived T_{reg}
WGS	Whole genome shotgun

2

CHAPTER

Enhancement of IFNγ production in CD4$^+$ T cells by distinct commensals ameliorates *Salmonella* induced disease[1]

2.1 Summary

The microbiota contributes to colonization resistance against invading pathogens directly through competition for metabolites and production of inhibitory substances, as well as indirectly by priming protective immune responses. However, the identities of commensal bacteria that modulate host resistance and immune-mediated protection remain largely elusive. Using isogenic mouse lines with distinct microbiota profiles, we demonstrate that severity of disease induced by enteric *Salmonella* Typhimurium infection is strongly modulated by microbiota composition in individual lines prior to infection. Transferring a restricted community of eleven cultivable intestinal commensals from protected into susceptible mice decreases tissue colonization of *S.* Typhimurium and consequently severity of disease. We identify that reduced weight loss and prolonged survival depend on microbiota-enhanced IFNγ production, primarily in CD4$^+$ T cells. In summary, we show that distinct microbiota members protect against intestinal *Salmonella* infection via induction of antibacterial IFNγ responses.

[1] Parts of the chapter have been published: Thiemann, S., Smit, N., Roy, U., Lesker, T. R., Gálvez, E. J. C., Helmecke, J., et al. (2017). Enhancement of IFNγ Production by Distinct Commensals Ameliorates Salmonella-Induced Disease. *Cell Host & Microbe*, *21*(6), 682–694.e5.

2.2 Introduction

The human intestine harbors a dense and diverse microbial ecosystem, termed microbiota, which includes bacteria, archaea and eukaryotes. Over the past decades it has been established that the microbiota and their metabolites contribute to the physiology of the host during health and disease (Clemente et al., 2012; Ley et al., 2008). In the context of infections the resident microbiota contributes to limit colonization of the intestine with enteric pathogens, to prevent tissue invasion as well as to ensure proper activation of the immune system (Buffie and Pamer, 2013). Microbiota-mediated protection against pathogens can derive directly from commensal-pathogen interactions (e.g. nutrient competition, production of inhibitory substances, delivery of toxic products), or indirectly by enhancing protective immune-mediated antibacterial pathways (Caballero and Pamer, 2015). Disturbance of the resident microbiota and thus, altered susceptibility to infections can be induced by changes in diet and intake of medication (David et al., 2014; Ubeda et al., 2010). In particular, antibiotic use is associated with an increased susceptibility to several enteric infections, including *Clostridium difficile* infections and non-typhoidal *Salmonella* disease, which is at least partly mediated by altering the composition and functionality of resident bacterial populations (Bohnhoff and Miller, 1962; Doorduyn et al., 2006; Doré et al., 2004; Ng et al., 2014; Pavia et al., 1990; Sekirov et al., 2008).

Non-typhoidal *Salmonella* (NTS) species, including *Salmonella enterica* spp. serovar Typhimurium (*S.* Typhimurium) belong to the family of Gram-negative Enterobacteriacea. Infections with NTS species are one of the leading causes of foodborne disease outbreaks and are the major bacterial cause of acute gastroenteritis, causing approximately 93.8 million cases and resulting in 155,000 deaths per year (Majowicz et al., 2010). Upon ingestion with contaminated food, *Salmonella* has to compete with the resident intestinal microbiota and cope with the mucosal immune system to colonize the host. While the mucosal inflammation is required to eventually clear infection, *Salmonella* intriguingly benefits from it to outgrow commensal bacteria; e.g., utilizing specialized metal-uptake systems and inflammation-associated metabolites (Raffatellu et al., 2009; Stecher et al., 2007; Winter et al., 2010).

Vice versa, commensals are able to confer resistance against *Salmonella*, e.g. via production of inhibitory substances such as organic acids (Sun and O'Riordan, 2013). Moreover, one study identified that commensals contribute to immune-mediated protection against *Salmonella* via induction of antimicrobial peptides in Paneth cells limiting invasion and systemic dissemination (Vaishnava et al., 2008). Despite these advances it is not fully understood yet how the intestinal microbiota primes the host mucosal inflammatory response to combat against *S.* Typhimurium infection.

Here, we employ mouse lines with distinct microbiota compositions and a mouse model of *Salmonella*-induced diarrhea that requires antibiotic induced disruption of direct colonization resistance. We demonstrate that severity of disease induced by *S.* Typhimurium is strongly dependent on the composition of the intestinal microbiota. We identify that resident intestinal bacteria from protected mice present before antibiotic treatment induce enhanced production of IFNγ by $CD4^+$ T cells in the lamina propria and demonstrate that modulation of IFNγ production by the protective microbiota was necessary for augmented protection against *S.* Typhimurium. Finally, we show that transfer of 11 different bacterial species isolated from protected mice to susceptible mice recapitulated the protective effect by attenuating tissue invasion of *S.* Typhimurium. These results identify for the first time that modulation of IFNγ production by specific intestinal bacteria reduces the severity of *Salmonella*-induced disease and thus potentially guide the development of novel probiotic interventions against enteric infections.

2.3 Methods

2.3.1 Mice and microbiota manipulation by cohousing

Wild type, *Ifng*$^{-/-}$ (Dalton et al., 1993) and Foxp3^{tm1Flv}-IL-17$^{tm1.1Flv}$-IFNγ^{tm1Flv} (Gagliani et al., 2015) (all on C57BL/6N background) mouse lines were rederived to SPF-1 microbiota conditions by embryo transfer and bred at the animal facilities of the Helmholtz Centre for Infection Research (HZI) under enhanced specific pathogen-free (SPF) conditions (Stehr et al., 2009). C57BL/6N SPF-2, SPF-3, SPF-4 and SPF-5 were purchased from different vendors (Charles River, Janvier, NCI and Taconic) and housed at the Helmholtz Centre for Infection Research for at least two weeks before the start of the experiment. After the initial screen, C57BL/6N SPF-2 mice were bred at the HZI. Germ-free C57BL/6NTac mice were bred in isolaters (Getinge) in the germ-free facility at the HZI. For cohousing with microbiota donor mice, donor and recipient mice were housed for at least four weeks together before infection experiments. SFB-monocolonized NOD.CB17-*Prkdc*^{scid}/J mice were bred in gnotobiotic isolators at the Hannover Medical School, Germany. Colonization of SPF-1 mice with SFB was achieved by cohousing for at least four weeks before infection experiments. Presence of SFB was investigated in fecal sample by PCR with SFB specific primers (Barman et al., 2008). All mice were provided with sterilized food and water ad libitum. Mice were kept under strict 12h light cycle and housed in groups of up to 6 mice per cage. For infections experiments, we used age- and sex-matched mice between 10 and 14 weeks of age. Both female and male mice were used in experiments. All animal experiments have been performed in agreement with the local government of Lower Saxony, Germany.

2.3.2 Fecal transplantation and microbiota reconstitution

For fecal transplantation experiments, donor mice were euthanized, intestinal content was collected from large intestine, cecum and SI and pre-homogenized in BBL thioglycollate media (BD Bioscience) by vortexing. Under anaerobic conditions, fecal content was homogenized using a 70µm sterile filter. After centrifugation (10 min, 500 g, 4 °C), fecal material was

resuspended in BHI medium (Sigma-Aldrich). A total of 200 µL of fecal transplant was given by oral gavage to recipient mice, which were starved 2 hours prior. Control mice were given 200 µl of BHI medium. Four weeks after cohousing or fecal transplant feces were collected for 16S rRNA sequencing and gastrointestinal colitis was induced by infection with *S.* Typhimurium. For monocolonization experiments, germ-free C57BL/6 mice were housed in specific ventilated isocages (Techniplast). Mice were monocolonized with 200 µl *E. coli*. One week after monocolonization, gastrointestinal colitis was induced by infection with *S.* Typhimurium.

2.3.3 Isolation of fecal bacteria

Fecal content of donor mice was collected, transferred to BBL thioglycollate media (BD Bioscience) and weighted. Fecal content was pre-homogenized by vortexing. Under anaerobic conditions, fecal content was passed through a 70µm cell strainer and diluted to a concentration of 40mg/ml. Bacteria were cultured strictly anaerobically at 37°C for 2 days in an anaerobic chamber (Coy Laboratory) with following gas mix: 70% nitrogen, 20% CO_2 and 10% hydrogen. Bacteria were isolated by using the most probable number (MPN) technique (Goodman et al., 2011). Specifically, in a 96-well plate homogenized sample was diluted in a range that maximal 30% of wells showed detectable growth. After 2 days, wells showing growth were mixed and then transferred into recipient mice (200 µl/mouse). SM medium was used for isolation and dilution processes: consists of 37g/L BHI (Sigma-Aldrich), 1% vitamins supplement (ATCC MD-VS), 1% mineral supplement (ATCC MD-VS), 1% non essential amino acids (Biowest), 1mg of menadione, 0.5g/L L-Cysteine HCl and 10% FCS, 4.2 mg/L Histidine, 1.2 mg/L Hematin and 1mg/L Resazurin (all Sigma-Aldrich).

To identify the isolated bacteria, DNA was isolated via hot shot lysis: Hot shot lysis buffer (25mM NaOH and 0,5M EDTA in 500 mL dH_2O, pH 12) was added to bacterial culture and mixture was heated for 45-60 min at 95ºC. Subsequently, neutralization buffer (3.2 g Tris-HCl in 1L dH_20, pH 5) and 0.5 x TAE (10mL of 50x TAE in 1L dH_2O) buffer was added. 16S rRNA gene was amplified using specific primer pairs: 27F (5’ AGAGTTTGATCMTGGCTCAG)

and 1492R (5' TACGGYTACCTTGTTACGA CTT). The amplification program consisted of one cycle at 98°C for 3 min, followed by 30 cycles at 98°C for 10 s, 55°C for 30 s and 72°C at 1 min 30 s. DNA was purified using MiniElute 96 UF PCR Purification Kit (Qiagen) and subsequently sent to Sanger sequencing.

2.3.4 Colitis model induced by infection with *S.* Typhimurium

Naturally streptomycin-resistant *S. enterica* serovar Typhimurium SL1344 was used for infection experiments. Wild-type strain EM774 harbored an ampicillin-resistant plasmid and was used for infection experiments with ampicillin pre-treatment. Wild-type strain EM1046 contained a chromosomally integrated *luxCDABE* cassette, which confers kanamycin resistance (PMID 19123992) and was used for infection experiments with streptomycin pre-treatment. SL1344 strains EM951 and EM952 are deleted for the *spi-1* encoded injectisome (Δ*invH-sprB*::Frt-Chloramphenicol-Frt) or *spi-2* encoded injectisome (Δ*sseA-ssaU*::Frt-Chloramphenicol-Frt), respectively, and harbor a chloramphenicol-resistance cassette. *Salmonella* strains were grown overnight at 37°C in lysogeny broth (LB), with either 50 µg/ml kanamycin or 100 µg/ml ampicillin, then, diluted 1:100 in fresh medium, and subcultured for 4 hours. Bacteria were washed twice in ice-cold phosphate-buffered saline (PBS) and then used for infection experiments.

Water and food were withdrawn for 4 hours before mice were treated with 20 mg / mouse of streptomycin by oral gavage (o.g.). Afterwards, mice were supplied with water and food ad libitum. 20 hours after streptomycin treatment, water and food were withdrawn again 4 hours before the mice were infected with 10^5 CFU of *S.* Typhimurium in 200 µl PBS. Drinking water ad libitum was supplied immediately and food 2 hours post infection (p.i.). Mice were weighted every day and survival was monitored.

2.3.5 Analysis of bacterial loads in feces

Fresh fecal samples were collected and weighted. Samples were homogenized in 1ml LB media by bead-beating with 1mm zirconia/silica beads twice for 25 sec using a Mini-Beadbeater-96 (BioSpec). To determine

CFUs, dilutions of homogenized samples were plated on LB and MacConkey plates, if necessary 50 µg/ml Kanamycin was added to media.

2.3.6 Analysis of bacterial loads in fecal content and systemic organs

All mice were euthanized by asphyxiation with CO_2 and cervical dislocation at indicated time points. Intestinal tissues (small intestine, cecum, colon) and organs (mesenteric lymph nodes, spleen) were removed aseptically. To collect fecal content, organs were flushed with PBS. Organs were opened longitudinally, cleaned thoroughly with PBS and weighted. Organs and content were homogenized in PBS using a Polytron homogenizer (Kinemtatica). Dilutions of homogenized samples were plated on LB plates containing 50 µg/ml Kanamycin to determine CFUs.

2.3.7 ELISA and Multiplex Analysis

Intestinal tissues of mice infected with *S.* Typhimurium were washed and transferred into protease inhibitor (Complete, Roche) containing NP40 buffer at indicated time-points. The tissue was disrupted mechanically using bead-beating four times for 1 min with 1mm zirconia/silica beads. Sample were stored at -80°C until analysis. Concentrations of cytokines were measured using commercial ELISA kit for IL-6 (BioLegend) and multiplex assay for all other cytokines (ProcartaPlex, eBioscience). To normalize protein concentration, Micro BCA protein assay Kit (Thermo Scientific) was used. ELISA, multiplex and BCA protein kit were used according to manufacturer's instructions.

2.3.8 Isolation of lymphocytes and flow cytometry

To isolate lymphocytes of the lamina propria, density gradient centrifugation using Percoll was done as previously described (Nagano et al., 2012). Briefly, tissues were collected 20h p.i. and fecal content as well as Peyers patches in the SI were carefully removed. Tissues were opened longitudinally, organs were cleaned with PBS and then shaken in HBSS containing 2 mM EDTA for

20 min at 37°C. Tissues were cut into small pieces and incubated with digestion solution (RPMI 1640 containing 1% fetal bovine serum (FBS), 0.25 mg/ml collagenase D, 0.5 U/ml dispase and 5 µg/ml DNase I) in a shaker for 20 min at 37°C. Digested tissues were filtered through 70uM cell strainer (Falcon) and DMEM + 5% FBS was added to inactivate enzymes. The last two steps were repeated until all tissue was digested. After centrifugation, cells were resuspended in 4 ml of 40% Percoll (GE Healthcare) and overlaid on 4 ml of 80% Percoll. Percoll gradient separation was performed by centrifugation at 450 g for 25 min at 25°C. Cells in the interphase were collected and used as LP lymphocytes. The collected cells were then suspended in staining buffer containing PBS, 1% FBS and 2 mM EDTA. The following antibodies were used: AlexaFluor-700 labeled anti-CD45 (30-F11), APC labeled anti-TCRgd (GL3), BV650 labeled anti-CD3 (17A2), BV785 labeled anti-CD4 (RM4-5) (Biolegend). To distinguish live dead cells AlexaFluor-350 NHS Ester (Life Technologies) was used. Flow cytometry analysis was performed using a BD LSRFortessa (BD Biosciences) and FlowJo software (TreeStar Inc.).

2.3.9 DNA isolation and 16S rRNA analysis

Fresh stool samples of mice were collected and immediately stored at -20°C. DNA was extracted according to established protocols using a method combining mechanical disruption (bead-beating) and phenol/chloroform-based purification (Turnbaugh et al., 2009). Briefly, sample was suspended in a solution containing 500µl of extraction buffer (200 mM Tris, 20mM EDTA, 200mM NaCl, pH 8.0), 200µl of 20% SDS, 500µl of phenol:chloroform:isoamyl alcohol (24:24:1) and 100µl of 0.1 mm zirconia/silica. Samples were homogenized twice with a bead beater (BioSpec) for 2 min. After precipitation of DNA, crude DNA extracts were resuspended in TE Buffer with 100µg/ml RNase and column purified to remove PCR inhibitors (BioBasic). Amplification of the V4 region (F515/R806) of the 16S rRNA gene was performed according to previously described protocols (Caporaso et al., 2011). Samples were sequenced on an Illuminia MiSeq platform (PE250). Filtering of sequences for low quality reads and barcode-based binning was performed by using QIIME

v1.8.0 (Caporaso et al., 2010). Reads were clustered into 97% ID OTUs using Open-reference OTU picking with UCLUST (Edgar, 2010) followed by taxonomic classification using the RDP Classifier executed at 80% bootstrap confidence cut off (Wang et al., 2007). Sequences without matching reference dataset, were grouped as *de novo* using UCLUST. Phylogenetic relationships between OTUs are determined using FastTree to the PyNAST alignment (Price et al., 2010). The OTU absolute abundance table and mapping file are used for statistical analyses and data visualization in the R statistical programming environment package phyloseq (McMurdie and Holmes, 2013). To determine bacterial OTUs that explained differences between microbiota settings, linear discriminant analysis (LDA) effect size (LEfSe) method were used (Segata et al., 2011). OTUs with Kruskal-Wallis test <0.05 and LDA scores >4.0 were considered informative.

2.3.10 Evolutionary relationships of taxa

Phylogenetic tree was created using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.37487100 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) is shown next to the branches (Zharkikh and Li, 1995). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

2.3.11 Statistical analysis

Data were analyzed with GraphPad Prism v6.0 and R statistical programming environment. Data are expressed as mean ± SEM. Differences were analyzed by Student's t test and ANOVA. *P* values indicated represent a non-parametric Mann-Whitney U test or Kruskal-Wallis test comparison of totals between groups. *P* values lower than 0.05 were considered as significant: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

2.4 Results

2.4.1 Isogenic mouse lines feature different susceptibility towards *S.* Typhimurium infection

It has been noted that the composition of the intestinal microbiota is associated with the degree of colonization resistance and severity of gastrointestinal disease during enteric *S.* Typhimurium infection, however the identity of bacteria contributing to this phenotype has largely remained unknown (Stecher et al., 2010). In order to identify specific bacteria that modulate *Salmonella* infection, we investigated susceptibility to disease induced by oral infection with *S.* Typhimurium using isogenic mouse lines with distinct composition of the microbiota. Age- and gender-matched C57BL6/N mice were obtained from different specific pathogen free (SPF) breeding facilities (SPF-1 – SPF-3) and housed in our vivarium for at least two weeks to allow adjustment to environmental conditions such as diet. Subsequently, mice were pretreated with a single dose of streptomycin and infected 24 hours later with *S.* Typhimurium (10^5 CFU) by oral gavage (Figure 2.1 A).

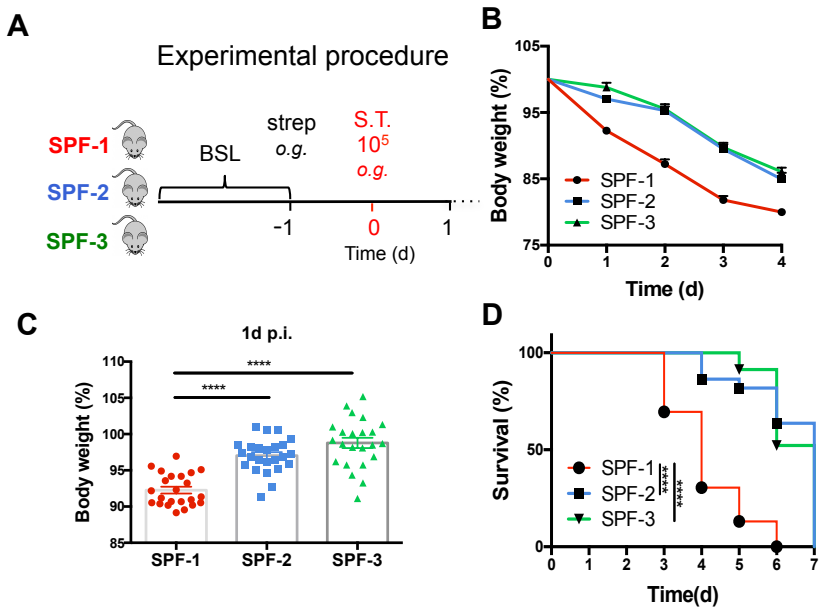

Figure 2.1 - Isogenic mouse lines from different vendors show distinct susceptibility during *S.* Typhimurium infection. (A) SPF-1, SPF-2 and SPF-3 Mice were treated with streptomycin (strep) 1 day prior infection and infected *o.g.* with 10^5 CFU *Salmonella* Typhimurium (S.T.). (B-D) Body weight was recorded during infection (B) and body weight of individual mice on day 1 p.i. is shown (C). Survival of mice was examined (D). Results represent n=6-25 mice/group as mean ± SEM from at least two independent experiments. *P* values indicated represent a Mann-Whitney U test comparison of totals between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

SPF-2 and SPF-3 mice displayed in contrast a lower disease severity upon infection compared to SPF-1 mice as demonstrated in decreased weight loss and prolonged survival (Figure 2.1 B-D).

First, we performed 16S rRNA gene amplicon sequencing from fecal DNA at baseline before antibiotic treatment to compare the microbiota composition. Analysis of β-diversity revealed a significant difference between the microbial communities of SPF-1, SPF-2 and SPF-3 mice with samples clustering distinctively according to the mouse line, respectively (Figure 2.2 A).

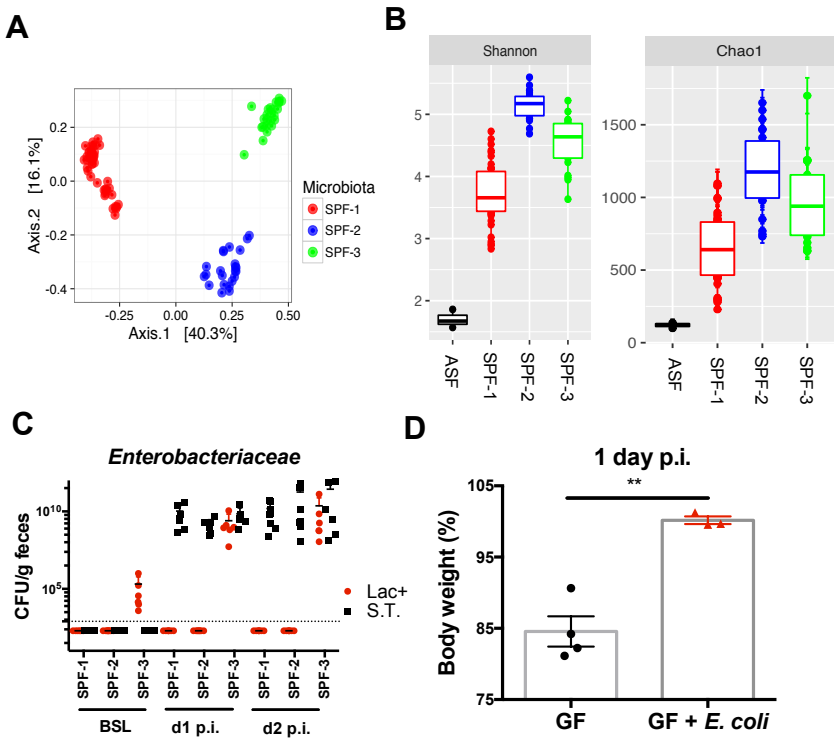

Figure 2.2 - Isogenic mouse lines from different vendors reveal distinct microbial communtiy. (A and B) Fecal microbiota of SPF-1, SPF-2 and SPF-3 mice was analyzed at steady state (BSL) using 16S rRNA analysis. Analysis of β-diversity using Bray-Curtis dissimilarity matrix and PCoA plot (A) as well as α-diversity using Shannon and Chao1 index are displayed (B). (C) Fecal samples prior infection as well as 1 day p.i. and 2 days p.i. were cultured on MacConkey agar with and without kanamycin. Lactose-fermenting (Lac+) colonies represent *Escherichia coli.* Non-lactose-fermenting bacteria (Lac-) colonies represent S.T. (E) *Escherichia coli*, isolated of SPF-3, was transferred in germ-free mice and body weight was analyzed 1 day p.i. Results represent n=3-8 mice/group from one experiment (C, D) or from at least two independent experiments (A, B) as mean ± SEM. Dashed line indicate the limit of detection. *P* values indicated represent a Mann-Whitney U test comparison of totals between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Notably, α-diversity varied between the mouse lines and specifically, SPF-1 mice had lower Shannon (species richness combined with abundance and evenness) and Chao1 indices (species richness) than SPF-2 and SPF-3 mice

(Kruskal-Wallis rank sum test, p-value= 3.387e-14) (Figure 2.2 B). Nonetheless, SPF-1, SPF-2 and SPF-3 mice all displayed a high degree of complexity compared to the Altered Schaedler Flora (ASF), a low diversity community containing a mixture of only 8 bacterial strains and thus, can be consequently considered as high-complexity communities (Dewhirst et al., 1999).

Commensal Enterobacteriaceae (e.g. *Escherichia coli*) have been identified as competitors of invading *S.* Typhimurium (Deriu et al., 2013; Lupp et al., 2007; Stecher et al., 2007). To analyze whether Enterobacteriaceae are present in our mouse lines, we quantified the level of Enterobacteriaceae before and during infection by culturing feces on MacConkey agar that enriches Gram-negative enteric bacilli and enables differentiation of colonies according to the ability to ferment lactose. Non-lactose fermenting colonies (Lac-), which were absent in all mouse lines before infection, appeared after infection and presumably consisted exclusively of *S.* Typhimurium as suggested by equal CFU numbers on *S.* Typhimurium-selective kanamycin-containing MacConkey plates (Figure 2.2 C, data not shown). Despite the differences in weight loss between mouse lines, comparable numbers of *S.* Typhimurium CFUs were detected in feces collected from SPF-1, SPF-2 and SPF-3 mice (Figure 2.2 C). Lactose-utilizing colonies (Lac+) were detected routinely at low numbers (10^5 CFU/g feces) in SPF-3 mice before infection, but were absent in SPF-1 and SPF-2 mice. During infection Lac+ colonies expanded in SPF-3 mice to comparable numbers as *S.* Typhimurium (Figure 2.2 C) and were not detected in SPF-1 and SPF-2 mice. We isolated the Lac+ colonies of SPF-3 mice and identified them by Sanger sequencing of their 16S rRNA gene as *Escherichia coli*. To examine if *E. coli* recovers the resistant SPF-3 phenotype, we transferred the isolated *E. coli* strain of SPF-3 mice into germ-free (GF) mice and induced *S.* Typhimurium disease according to Figure 2.1 A. Analyzing body weight 1 day p.i. revealed that gnotobiotic mice solely colonized with *E. coli* showed a lower susceptibility to *S.* Typhimurium (Figure 2.2. D). Our results demonstrate that SPF-2 mice are in contrast to SPF-3 mice devoid of Enterobacteriaceae prior to infection and did not show any enterobacterial bloom, which affects *Salmonella* infection.

Hence we conclude that protection seen in SPF-2 mice is independent of Enterobacteriaceae, but dependent on yet unidentified commensal bacteria.

2.4.2. Altered susceptibility to *S.* Typhimurium gastroenteritis is mediated by SPF-2 microbiota

To formally exclude the possibility that genetic differences between SPF-1 and SPF-2 mice as a result of being derived from different C57BL6/N sub-lines or acquired by spontaneous mutation during inbreeding are responsible for the phenotype, we cohoused SPF-1 and SPF-2 mice with germfree C57BL6/NTac mice for 4 weeks before infection (Figure 2.3 A). 16S rRNA sequencing confirmed that recipient exGF mice harbored a similar microbiota composition as their respective SPF-1 or SPF-2 donor after four weeks of cohousing (Figure 2.3 B).

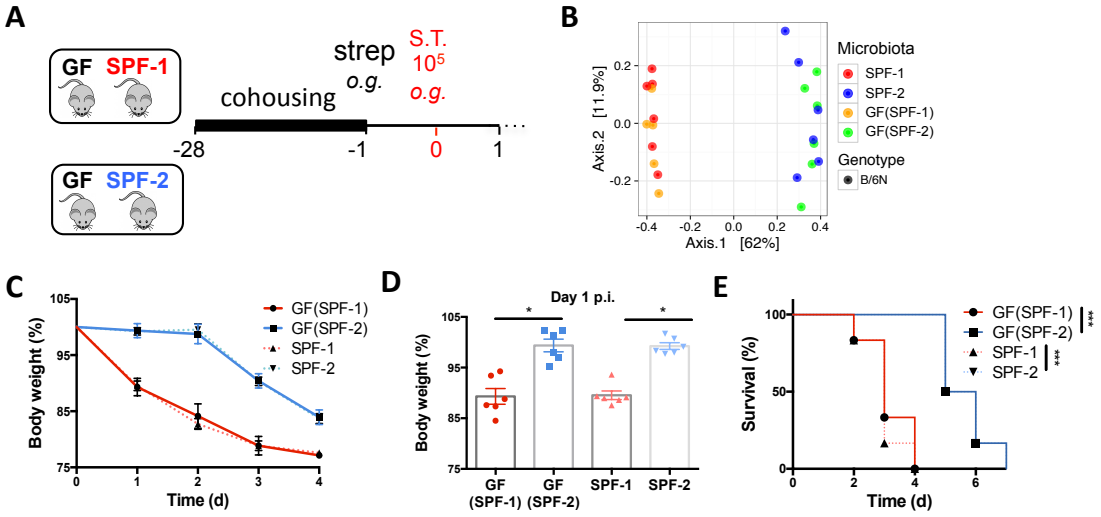

Figure 2.3 - Differences in disease severity are microbiota-driven. (A) C57BL6/NTac germ-free mice (GF) were cohoused with SPF-1 and SPF-2 for 4 weeks and infected with S.T. (B) Fecal microbiota of single-housed (SPF-1, SPF-2) and cohoused (GF(SPF-1), GF(SPF-2)) mice were analyzed after 4 weeks of cohousing using 16S rRNA analysis including Bray-Curtis dissimilarity matrix and PCoA plot. (C-E) Body weight was measured over time of infection (C) and significances in body weight between single-housed and cohoused were analyzed 1 day post infection (p.i.) (D). Survival of mice was examined during the infection (E). Results represent one independent experiment n=5-6 mice/group as mean ± SEM. *P* values indicated represent a Mann-Whitney U test comparison of totals between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Strikingly, weight loss and survival of the recipient GF mice after infection with *Salmonella* were identical to the respective cohousing partners, indicating that

differences in disease severity are indeed microbiota-driven (Figure 2.3 C-E). Next, we examined how mixing of these microbial communities influenced the protective and susceptible phenotypes. Therefore, SPF-1 and SPF-2 mice were cohoused to allow exchange of the microbiota between mouse lines (Figure 2.4 A).

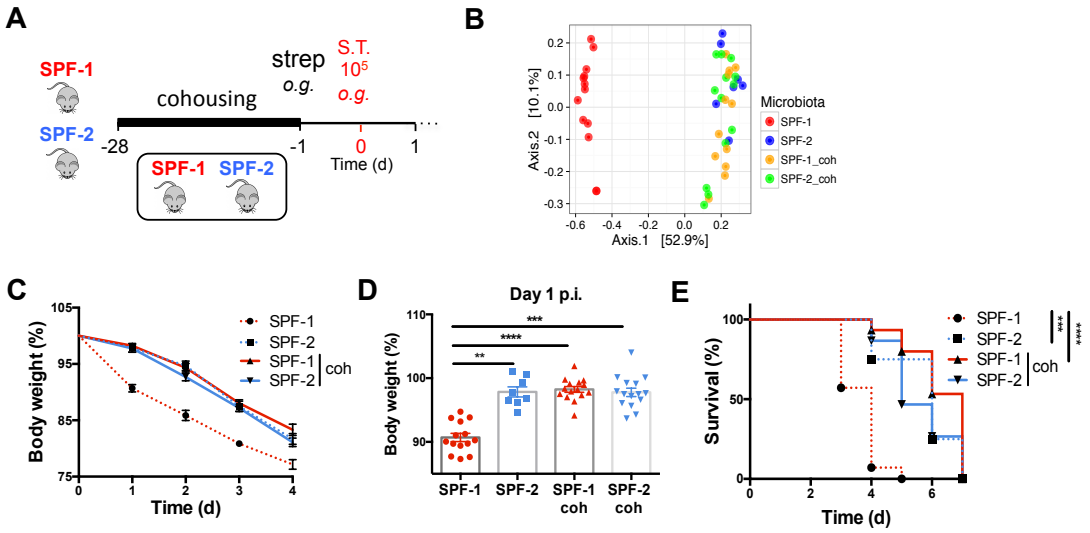

Figure 2.4 - Protective phenotype is transferable by cohousing. (A) SPF-1 and SPF-2 were cohoused for 4 weeks and infected with S.T. (B) Fecal microbiota of single-housed (SPF-1, SPF-2) and cohoused (SPF-1 coh, SPF-2 coh) mice was analyzed after 4 weeks of cohousing using 16S rRNA analysis including Bray-Curtis dissimilarity matrix and PCoA plot. (C-E) Body weight of single-housed and cohoused mice was recorded during infection (C) and body weight of individual mice on day 1 p.i. is displayed (D). Survival of mice was examined (E). Results represent n=6-14 mice/group from at least two independent experiments as mean ± SEM. *P* values indicated represent a Mann-Whitney U test comparison of totals between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Notably, 16S rRNA sequencing revealed that upon cohousing the microbiota in SPF-1 and SPF-2 mice largely resembled the microbiota in SPF-2 mice before cohousing (Figure 2.4 B). Comparison of body weight loss and survival between cohoused and single-housed mice showed that SPF-1 mice cohoused with SPF-2 mice (SPF-1 coh) phenocopied SPF-2 mice further corroborating that specific components of the SPF-2 microbiota are responsible for decreased severity of *S.* Typhimurium-induced disease (Figure 2.4 C-E). To analyze if this phenotype could also be transferred by fecal transplantation, SPF-1 mice were transferred with homogenized fecal content of SPF-2 mice (donor) by oral gavage (Figure 2.5 A). Analysis of microbial composition after 4 weeks revealed a similar microbiota in SPF-1 mice, which received a fecal transplant of SPF-2 mice, compared to SPF-2

mice (Figure 2.5 B). In addition, SPF-1 mice, which received a fecal transplant from SPF-2 mice, revealed a similar disease progression during *Salmonella* infection compared to SPF-2 mice showing diminished body weight loss and higher survival rate (Figure 2.5 C-D). These results show that it is indeed the SPF-2 microbiota that is able to protect against *S.* Typhimurium.

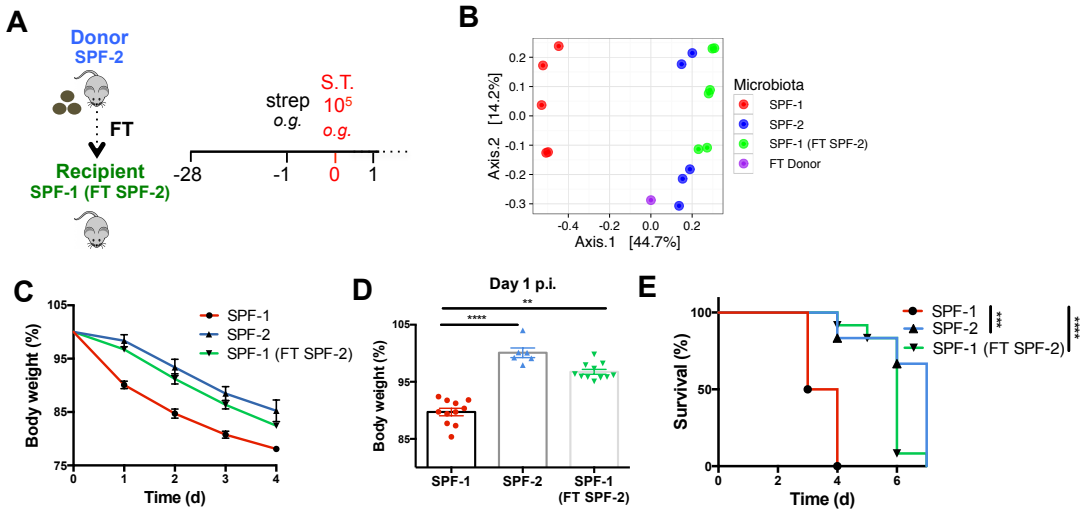

Figure 2.5 - Protective phenotype is transferable by fecal transplantation. (A) Fecal content of SPF-2 was transferred by fecal transplant (FT) to SPF-1 (FT SPF-2) mice and mice were infected after 4 weeks with S.T. (B) Fecal microbiota was analyzed after 4 weeks of FT by 16S rRNA analysis using Bray-Curtis dissimilarity matrix and PCoA plot. (C-E) Body weight of mice was recorded during infection (C) and body weight of individual mice on day 1 p.i. is displayed (D). Survival of mice was examined (E). Results represent n=6-14 mice/group from at least two independent experiments as mean ± SEM. *P* values indicated represent a Mann-Whitney U test comparison of totals between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

2.4.3 Microbiota mediated protection is associated with decreased tissue colonization and independent of direct colonization resistance

After oral infection *S.* Typhimurium first colonizes the intestinal lumen, where it competes with the commensal microbiota (Kaiser et al., 2012). Subsequently, *S.* Typhimurium infects epithelial cells and invades the lamina propria triggering intestinal inflammation. Finally, *S.* Typhimurium spreads via the lymphatic vessels to the mesenteric lymph nodes and then into other systemic organs as spleen and liver resulting in lethal systemic infection in susceptible hosts (Barthel et al., 2003). To start dissecting how the microbiota in SPF-2 mice ameliorates disease, we quantified *Salmonella* colonization in the intestinal lumen and tissue (6, 20, and 44 hours p.i.). Comparing SPF-1

and SPF-2 mice we detected similar numbers of CFUs in the lumen of gastrointestinal regions at all analyzed time points (6, 20, and 44 hours p.i.) (Figure 2.6 A-H). Unlike the comparable *Salmonella* loads in the lumen of SPF-1 and SPF-2 mice, we detected lower numbers of CFUs in the tissue of SPF-2 mice compared to SPF-1 mice; up to 100-fold lower numbers of CFUs in the SI already at all analyzed time points and five-fold differences in the cecum after 20 hours p.i. (Figure 2.6 B, 2.6 E). In the SI the largest difference was observed in the terminal ileum (Figure 2.6 H). Based on these results, we conclude that severity of disease correlates not with the luminal colonization of *S.* Typhimurium, but rather with higher pathogen invasion.

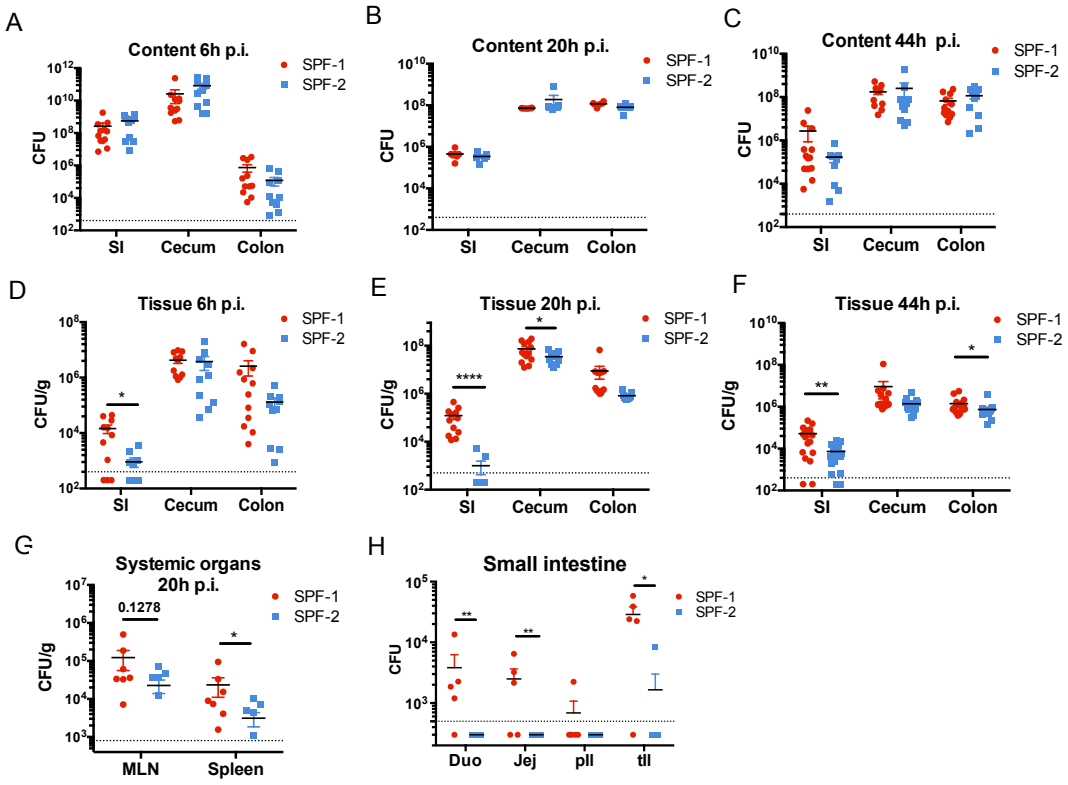

Figure 2.6 - Bacterial load of *S.* Typhimurium at different time points. (A-G) *Salmonella* infected mice were sacrificed 6 hours p.i. (A, D), 20 hours p.i. (B, E, G) and 44 hours p.i. (C, F). Number of *S.* Typhimurium CFUs was determined in luminal content (A-C) and tissue (D-F) of small intestine (SI), cecum and colon as well as in mesenteric lymph nodes (MLN) and spleen (G). (H) Mice were sacrified 6 hours p.i. and small intestine was divided in four same sized parts as indicated: Duodenum (Duo), jejunum (Jej), proximal ileum (pIl) and terminales ileum (tIl). Number of *S.* Typhimurium was determined. Results represent n=5-13 mice/group as mean ± SEM from one (H) or at least two independent experiments (A-G). Dashed line indicates the limit of detection. *P* values indicated represent a Mann-Whitney U test comparison of totals between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

S. Typhimurium is able to invade the host tissue and eventually induce intestinal inflammation via different routes, in which type III injectisome

systems encoded on either the *Salmonella* Pathogenicity Islands (SPI)-1 or -2 play an essential role (Hapfelmeier et al., 2005). SPI-1 is essential for induction of inducing uptake by enterocytes, while SPI-2 is required for survival and replication in phagocytic cells in the lamina propria. In order to identify whether induction of inflammation via any of the two pathways contributes to altered disease susceptibility, we infected SPF-1 and SPF-2 mice with wild type (WT) and D*spi-1* mutant *S.* Typhimurium. In contrast to infection with the WT strain, no difference in weight loss and survival was observed between SPF-1 and SPF-2 mice after infection with D*spi-1* mutant strain, indicating that induction of inflammation downstream of SPI-1, i.e. infection of enterocytes, is affected by the SPF-2 microbiota (Figure 2.7 A).

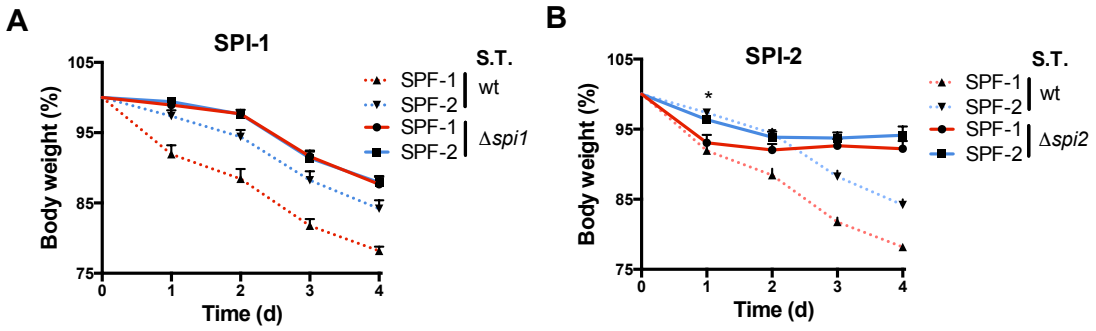

Figure 2.7 - Higher *S.* Typhimurium susceptibility correlates with increases tissue invasion via SPI-1 type III secretion system. (A and B) Body weight of mice infected with *S.* Typhimurium wild type (wt) as well as with *S.* Typhimurium deficient in SPI-1 (A) and SPI-2 (B) are shown. Results represent n=5-13 mice/group from at least two independent experiments as mean ± SEM. *P* values indicated represent a Mann-Whitney U test comparison of totals between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Notably, upon infection D*spi-2* mutant *S.* Typhimurium SPF-1 and SPF-2 mice displayed the same difference of weight loss between the mouse lines early during infection (day 1 p.i.) (Figure 2.7 B). As previously reported, mice subsequently recovered and did not succumb to infection (Raupach et al., 2003). These results strongly suggest that the microbiota in SPF-2 mice does not protect the host via direct competition with *S.* Typhimurium in the lumen but rather by limiting the ability of *S.* Typhimurium to induce intestinal inflammation via the SPI-1 encoded injectisome.

2.4.4 Microbiota mediated modulation of host's IFNγ production is crucial for colonization resistance

Already at early phases of the infection, distinct antimicrobial molecules and cytokines produced by the host act in concert for defense against *S.* Typhimurium (Kaiser et al., 2012). We hypothesized that differences in microbiota composition between SPF-1 and SPF-2 mice would result in distinct immune responses against *S.* Typhimurium infection that would explain the different colonization of intestinal tissues. Comparison of cytokine profiles in the cecum 20 hours after *S.* Typhimurium infection readily revealed several cytokines that were either upregulated in SPF-1 (IL-6, IL-1a and IL-17F) or SPF-2 mice (IFNγ, IL-27 and IL-23) (Figure 2.8).

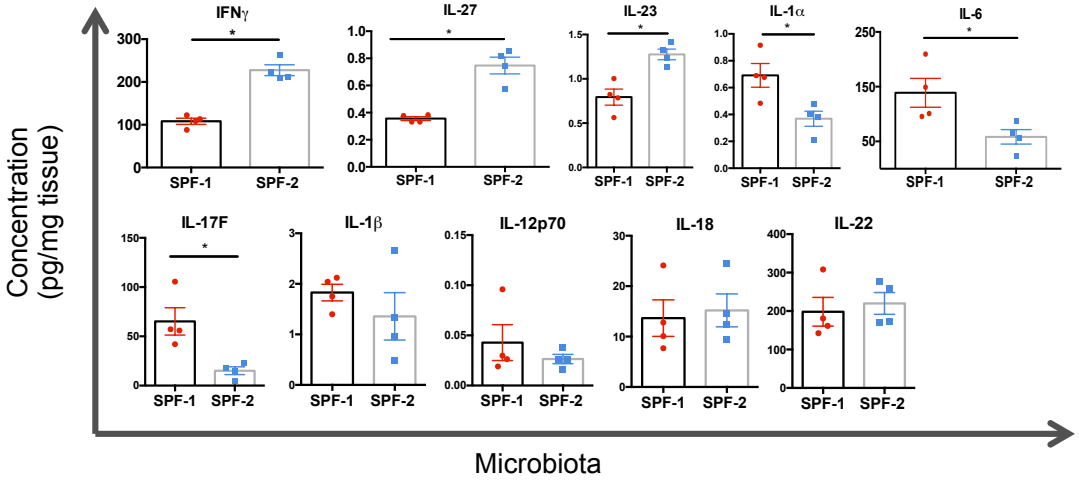

Figure 2.8 - Resident bacteria regulate distinct cytokine expression profile during *S.* Typhimurium infection. Streptomycin pretreated SPF-1 and SPF-2 mice were infected with 10^5 *S.* Typhimurium (S.T.) by oral gavage (*o.g.*). Mice were sacrificed 12 hours post infection (p.i.) and cecum was analyzed for interferon (IFN)-γ, interleukine (IL)-27, IL-23, IL-1α, IL-6, IL-17F, IL-1β, IL-12p70, IL-18, IL-22 by ELISA. Data derive from one experiment as mean ± SEM. *P* values indicated represent a Mann-Whitney U test between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Enhanced levels of IL-6 in SPF-1 mice may reflect the increased bacterial colonization in the tissue. In contrast, higher secretion of IFNγ, a cytokine that has been shown to contribute to host defense against *S.* Typhimurium (Rhee et al., 2005), specifically in SPF-2 mice, may reflect microbiota-modulated enhanced protective immune responses. In order to determine the cellular source of IFNγ during *Salmonella* infection, we employed a cytokine reporter mouse that allows for detection of IL-17 and IFNγ production *in vivo* by

monitoring the expression of fluorescent proteins in target cells by flow cytometry without manipulation *ex vivo*. These mice were bred harboring the SPF-1 microbiota and the SPF-2 microbiota was introduced by fecal transplantation analogous to Figure 2.5 A. In $CD3^-$ lymphocytes, including NK cells and ILCs, the frequency of IFNγ-producing cells was similar between SPF-1 and SPF-2 reporter mice in the cecum and SI (Figure 2.9 A-B).

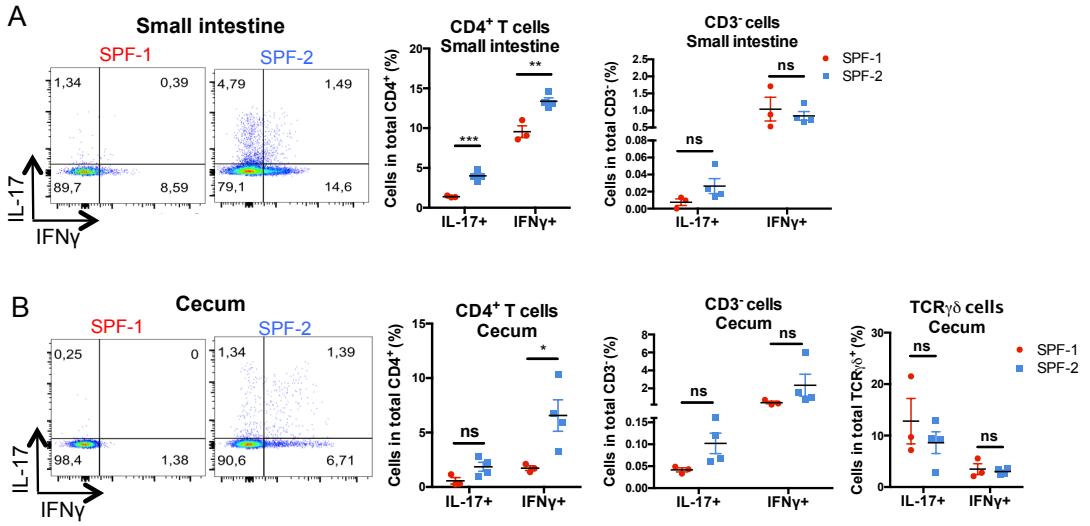

Figure 2.9 - Resident bacteria modulate IFNγ production by $CD4^+$ T cells. Reporter mice for IFNγ and IL-17 were rederived by embryo transfer and SPF-2 microbiota was transferred by fecal transplantation. Streptomycin pretreated mice were infected with 10^5 *S.* Typhimurium (S.T.) by oral gavage (*o.g.*). On day 1 p.i. cells were isolated from the lamina propria and analyzed by flow cytometry. Representative flow cytometry plots showing the frequency of $CD4^+$ T cell producing IFNγ and IL-17 in small intestine (A) and cecum (B). Graphs represent frequency (%) of $IFNγ^+$ and $IL\text{-}17A^+$ cells amongst $CD4^+$ cells, as well as $CD3^-$ and $TCRγδ^+$ cells in small inestine (A) and cecum (B). Data are shown for representative out of three independent experiments with n=3-4 mice per group as mean ± SEM. *P* values indicated represent unpaired t test comparison of totals between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns – not significant

However, a significant increase in the frequency of IFNγ-producing cells was detected in $CD3^+$ lymphocytes in SPF-2 mice, particularly of $CD4^+$ T cells but not $TCRγδ^+$ cells (Figure 2.9 A-B). Additionally, in SPF-2 mice increased frequencies of $CD4^+$ T cells in the SI, but not cecum, produced IL-17A (Figure 2.9 A-B). In order to test whether enhanced IFNγ production in SPF-2 mice contributes to the microbiota-mediated decrease in severity of *Salmonella*-induced disease, we decided to test the effect of the SPF2 microbiota in IFNγ deficient mice. To do so, we rederived *Ifng*$^{-/-}$ mice into the SPF-1 microbiota using embryo-transfer into foster mothers colonized with the SPF-1 microbiota. *Ifng*$^{-/-}$ mice with the SPF-2 microbiota were generated by fecal

transplantation as described in Figure 2.10 A. Importantly, comparison of microbiota composition of both WT and *Ifng*$^{-/-}$ mice with either the SPF-1 or SPF-2 microbiota, revealed no differences between the two genotypes (Figure 2.10 B). Strikingly, S. Typhimurium infected *Ifng*$^{-/-}$ mice harboring either the SPF-1 or the SPF-2 microbiota showed similar weight loss and survival, while control mice showed the same microbiota-dependent differences as previously described (Figure 2.10 C-E). This strongly suggests that the SPF-2 microbiota requires IFNγ to protect the host and that enhanced IFNγ production is derived from CD4^{+} T cells.

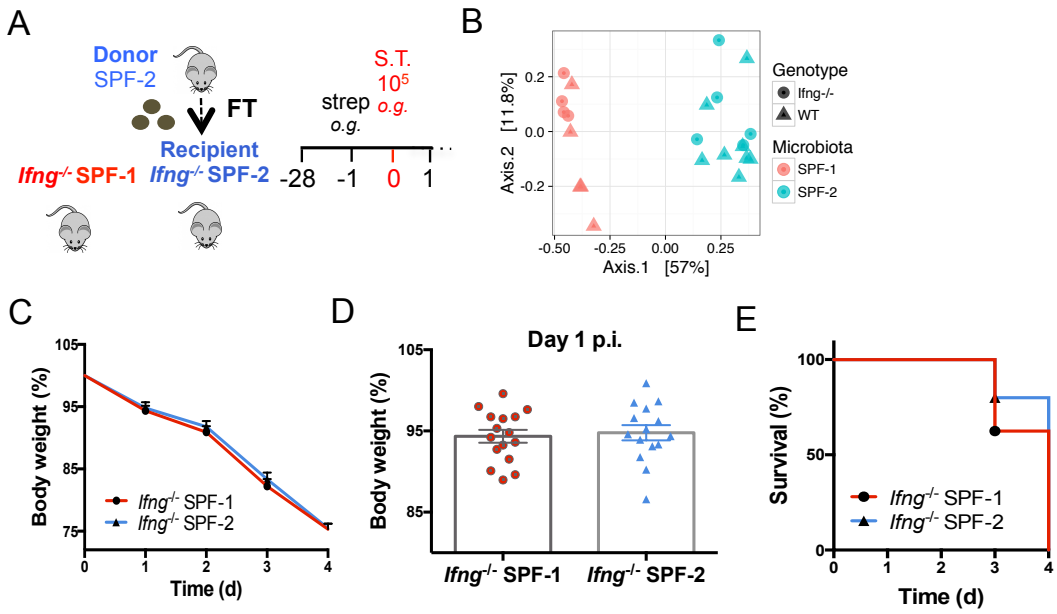

Figure 2.10 - Bacteria-modulated IFNγ is required for augmented protection towards *S.* Typhmurium. (A-E) *Ifng*$^{-/-}$ mice were rederived by embryo transfer (*Ifng*$^{-/-}$ SPF-1). SPF-2 microbiota was transferred by fecal transplantation (FT) (*Ifng*$^{-/-}$ SPF-2) and mice were infected 4 weeks later (A). Composition of the fecal microbiota was analyzed prior to streptomycin treatment by 16S rRNA analysis using Bray-Curtis dissimilarity matrix and PCoA plot (B). (C-E) Body weight (C) and survival (E) during course of infection are displayed as well as body weight of individual mice on day 1 p.i. is shown (D). Data are pooled with n=15-16 mice from two independent experiments as mean ± SEM. *P* values indicated represent a Mann-Whitney U test comparison of totals between groups.

2.4.5 Identification of microbial signatures that are linked with increased resistance towards *Salmonella* infection

In order to identify specific bacteria associated with resistance to *Salmonella* induced disease, we characterized the microbiota composition using 16S rRNA sequencing from fecal samples at baseline before the experiment

(BSL), after streptomycin treatment, and 1 day p.i. The microbial composition of SPF-1 and SPF-2 mice clustered differently before treatment, whereas it showed overall more similarity after streptomycin treatment and *S.* Typhimurium infection (Figure 2.11 A-B, 2.12).

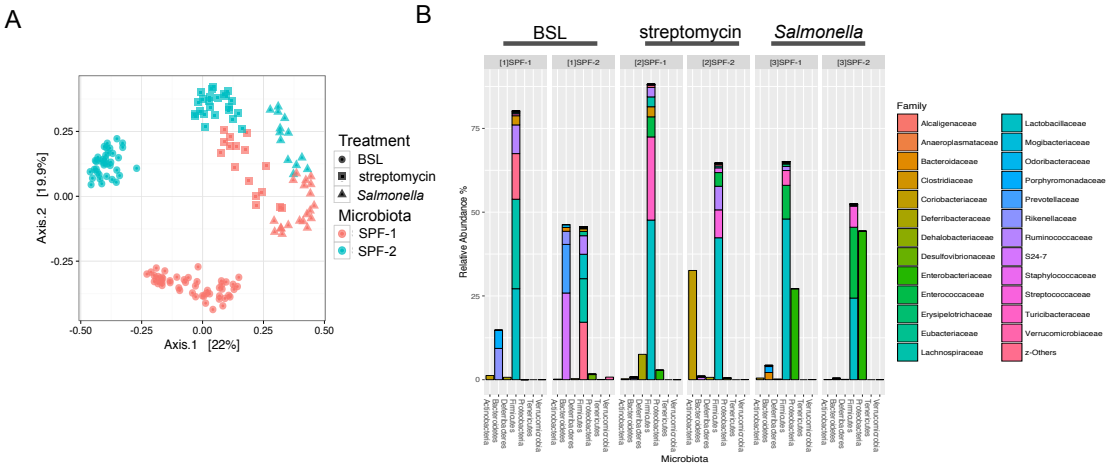

Figure 2.11 - Changes of the microbiota composition of SPF-1 and SPF-2 mice during infection. (A and B) Composition of the microbiota of SPF-1 and SPF-2 mice was analyzed by 16S rRNA analysis before treatment (BSL) (n=38-49), 1 day after streptomycin treatment (n=16-24) and 1 day p.i. with *S.* Typhimurium (n=14-19). Bray-Curtis dissimilarity matrix and PCoA plot demonstrate distances between communities at different time points (A). Relative abundances of bacterial families are shown and grouped according to their phylum; bars represent the mean of all mice within the group (B). Results represent more than 3 independent experiments.

Comparing the community in SPF-1 and SPF-2 mice using LEfSe (Segata et al., 2011), revealed that the SPF-1 microbiota at baseline is characterized by a significantly higher relative abundance of Firmicutes, including Clostridia (Families: Lachnospiraceae and Clostridiaceae) and Bacilli (Families: Lactobacillaceae and Turicibacteriaceae), but also Bacteroidia as Porphyromonadaceae and Rikenellaceae (Figure 2.13 A). SPF-2 mice featured a significantly higher relative abundance of Bacteroidetes (Families: S24-7 and Prevotallaceae) as well as with bacteria of the family Verrucomicrobiaceae. After streptomycin treatment, bacteria from Bacteroidales were diminished and the presence of bacteria from the family Coriobacteriaceae correlated with the protective phenotype of SPF-2 mice (Figure 2.12, 2.13 B).

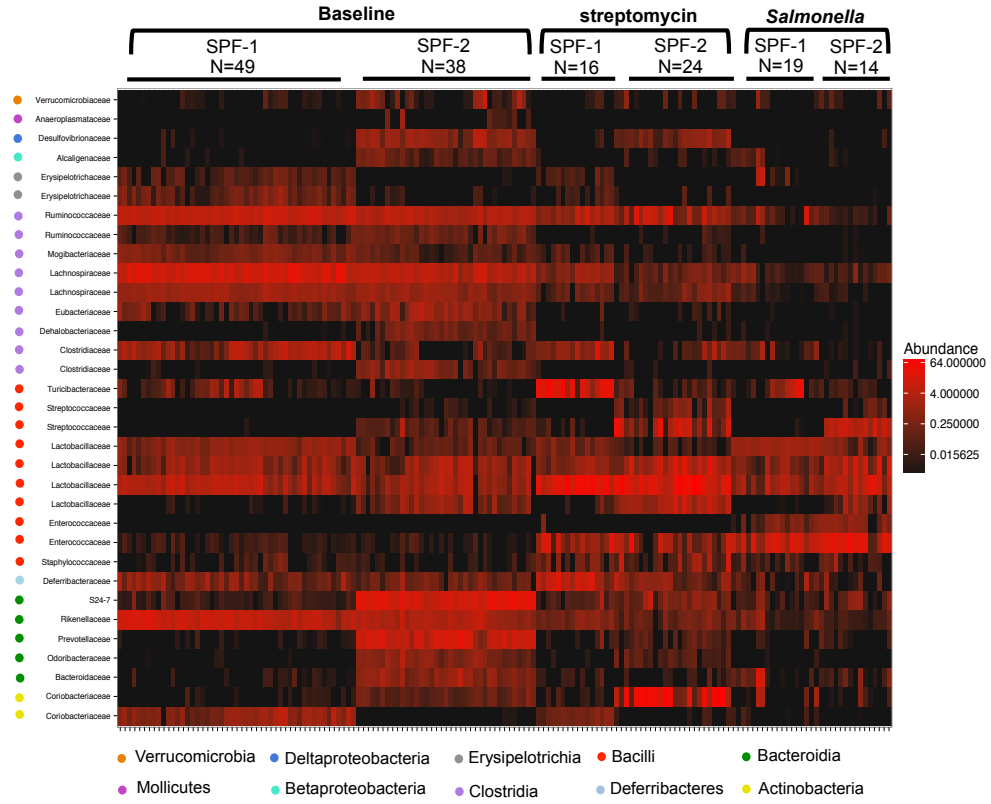

Figure 2.12 - Composition of intestinal microbiota at OTU level during different phases of *S.* Typhimurium infection. Heatmap of relative abundance of bacterial OTUs (≥97% sequence similarity, >0.05% relative abundance) sorted by family and class as well as SPF-1 and SPF-2 at baseline (n=38-49), after streptomycin treatment (16-24) and *Salmonella* infection 1 day p.i. (14-19). Results represent more than 3 independent experiments.

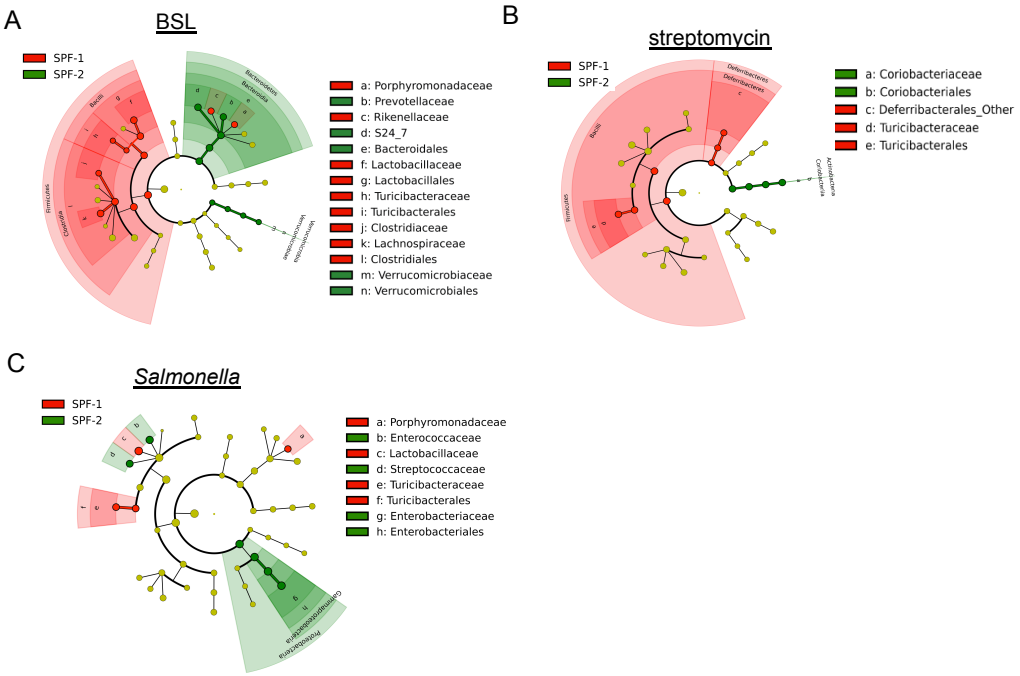

Figure 2.13 - Microbial signatures associated with amelioration of *S.* Typhimurium induced disease. Statistically significant differences between SPF-1 and SPF-2 before treatment (C), 1 day after streptomycin treatment (D) and 1 day p.i. (E) was analyzed using LEfSe (Kruskal-Wallis test $p<0.05$, LDA 4.0).

Moreover, 1 day p.i. we detected a significantly higher relative abundance of Enterococcaceae, Streptococcaceae and Enterobacteriaceae in SPF-2 mice (Figure 2.13 C). To investigate whether the resistant phenotype is linked to communities observed after pretreatment of mice with streptomycin, we pretreated SPF-1 and SPF-2 mice with ampicillin, which targets a different spectrum of bacteria. Strikingly, SPF-2 mice were protected relative to SPF-1 mice also using ampicillin as antibiotic pretreatment, despite large differences in the bacterial communities were observed after ampicillin compared to streptomycin treatment (Figure 2.14 A-C). These results suggest that members of the SPF-2 microbiota influence host resistance prior to antibiotic treatment.

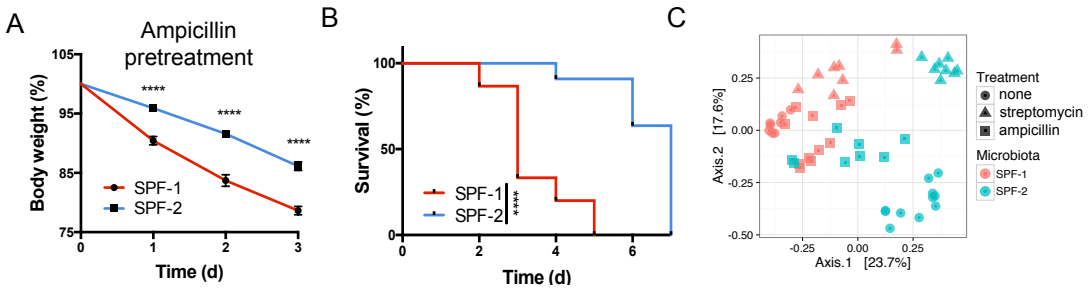

Figure 2.14 - Streptomycin and ampicillin treatment before infection reveals different microbiota composition but similar phenotype during infection. (A-C) Ampicillin pretreated SPF-1 and SPF-2 mice were infected with 10^5 *S.* Typhimurium (S.T.). Body weight (A) and survival (B) are shown. (C) Composition of the fecal microbiota of SPF-1 and SPF-2 was analyzed by 16S rRNA analysis before treatment (none), 1 day after streptomycin treatment and 1 day after ampicillin treatment. Bray-Curtis dissimilarity matrix and PCoA plot was used. Results represent n=11-15 mice/group as mean ± SEM pooled from two independent experiments. *P* values indicated represent a Mann-Whitney U test comparison of totals between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

2.4.6 Cultivable bacteria derived from the SPF-2 microbiota are responsible for the protective phenotype

Differentiation of $CD4^+$ T cells in the intestine is influenced by distinct members of the microbiota, e.g. induction of Th17 cells is supported by the presence of segmented filamentous bacteria (SFB) (Ivanov et al., 2009). Notably, it has also been reported that SFB increase IFNγ production from $CD4^+$ T cells (Gaboriau-Routhiau et al., 2009). Since we observed that the frequency of IL-17 and IFNγ-producing $CD4^+$ was increased in SPF-2 mice,

we investigated whether transfer of SFB into SPF-1 mice was able to reduce susceptibility to *S.* Typhimurium infection. Therefore, we cohoused SFB monocolonized donor mice with SPF-1 recipient mice for 4 weeks after which we confirmed that SFB successfully colonized SPF-1 mice (Figure 2.15 A). We then infected SPF-1 and SPF-1+SFB mice with *Salmonella* and analyzed body weight and survival. Compared to the SPF-2 microbiota containing SFB and other bacteria, transfer of SFB alone is unable to induce the protective phenotype (Figure 2.15 B-C).

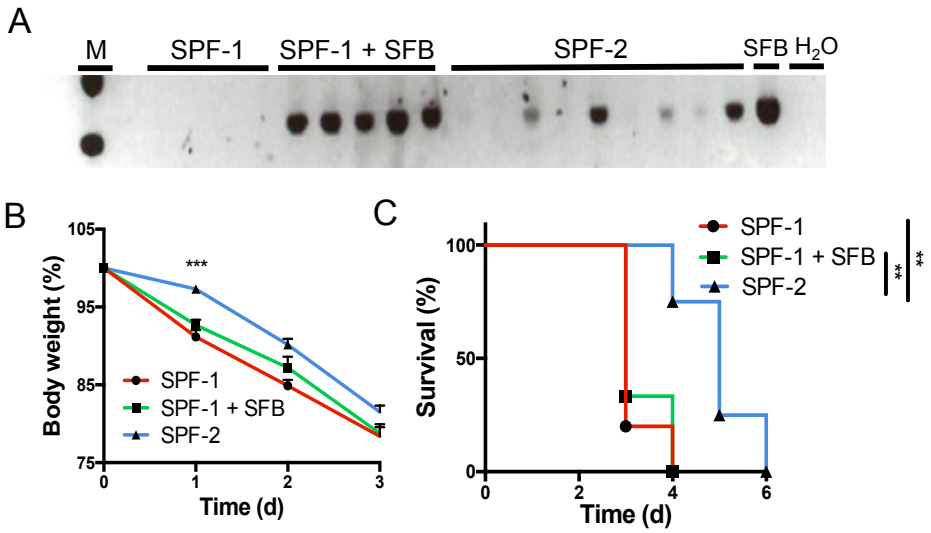

Figure 2.15 - Segmented filamentous bacteria (SFB) are dispensable for modulating protection to *S.* Typhimurium infection. (A-C) Monocolonized SFB mice were cohoused with SPF-1 mice (SPF-1 + SFB) for 4 weeks and mice were infected with S. Typhimurium. At baseline, abundance of SFB in mice was analyzed by PCR (A). Upon infection, body weight (B) and survival (C) was measured. Results represent n=4-15 mice/group of one experiments as mean ± SEM. *P* values indicated represent a Mann-Whitney U test comparison of totals between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

To identify bacteria able to transfer the protective SPF-2 phenotype, we cultured bacteria from the fecal content of SPF-2 mice (Goodman et al., 2011). Specifically, in a 96-well plate we diluted homogenized fecal content samples of SPF-2 mice in a range that maximal only 30% of wells showed a detectable growth after 2 days under anaerobic conditions (Figure 2.16 A). From one plate that showed growth in the desired range we identified the growing bacteria in each individual well by Sanger sequencing of the 16S rRNA gene. We recovered 11 different isolates from the following bacterial families: Prevotellaceae, Bacteroidaceae, S-24/7, Lactobacillaceae and Lachnospiraceae (Figure 2.16 B). In parallel, we transferred the content of

wells displaying growth to SPF-1 mice and after 4 weeks, analyzed the composition of the fecal microbiota using 16S rRNA sequencing. The microbiota composition in mice receiving the isolates was distinct from SPF-1 and SPF-1 mice which were transplanted with the SPF-2 community (Figure 2.16 C-D).

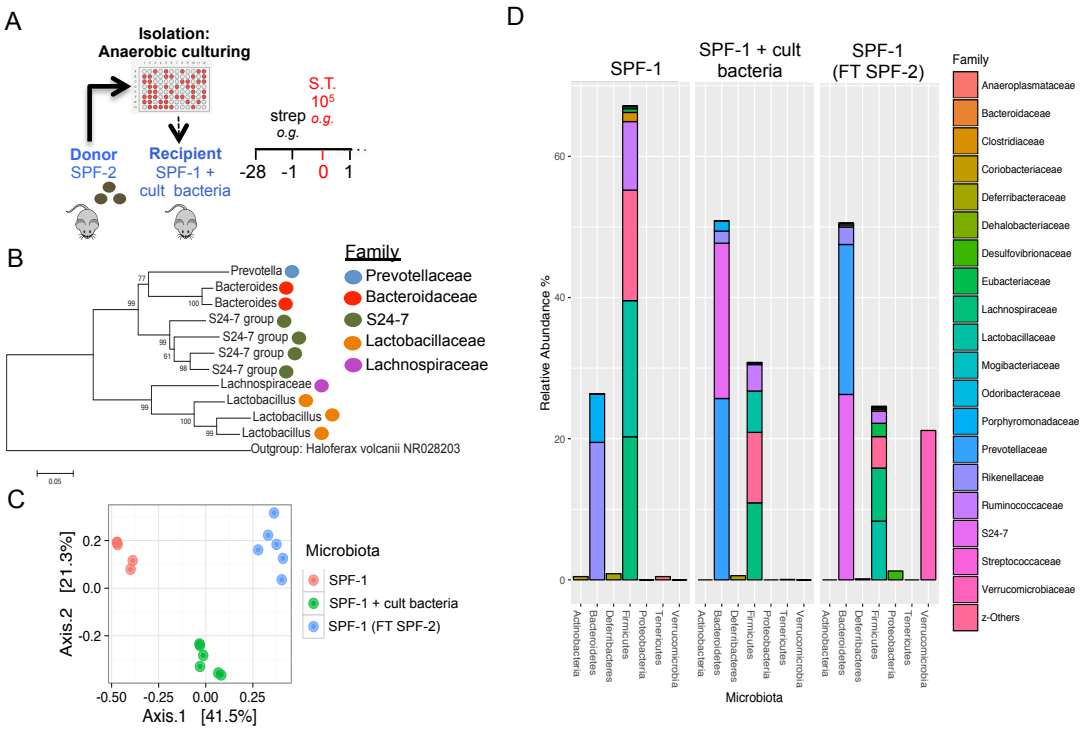

Figure 2.16 - Isolation of bacteria from fecal content of protected mice. (A-D) Culture collections were generated from fecal content of SPF-2 mice. Isolated bacteria were transferred to SPF-1 (SPF-1 + cult bacteria). As control whole SPF-2 community was transferred by fecal transplantation (SPF-1(FT SPF-2)). After 4 weeks mice were pretreated with streptomycin (strep) and infected with 10^5 CFU *S.* Typhimurium (S.T.) (A). (B) Phylogenetic tree of cultured bacteria of SPF-2 from full-length 16S rRNA gene sequences. (C) Fecal microbiota was analyzed after 4 weeks of transplantation by 16S rRNA analysis using Bray-Curtis dissimilarity matrix and PCoA plot. (D) Relative abundances of bacterial families are shown and grouped according to their phylum; bars represent the mean of all mice within the group. Results represent n=4-15 mice/group from one experiments.

Upon infection with *Salmonella* SPF-1 mice receiving the isolated bacteria had a similarly delayed disease progression as SPF-1 mice transplanted with the full SPF-2 community, as indicated by reduced body weight loss and enhanced survival compared to SPF-1 mice (Figure 2.17 A-C). In line with our previous results, *S.* Typhimurium loads in the contents of SPF-1 mice that received the cultured bacteria were similar to the one in SPF-1 mice, while pathogen loads in the tissues were reduced (Figure 2.17 D-E). These results

demonstrate that the mix of eleven isolated bacteria is able to ameliorate *Salmonella*-induced disease similar to the complex SPF-2 community.

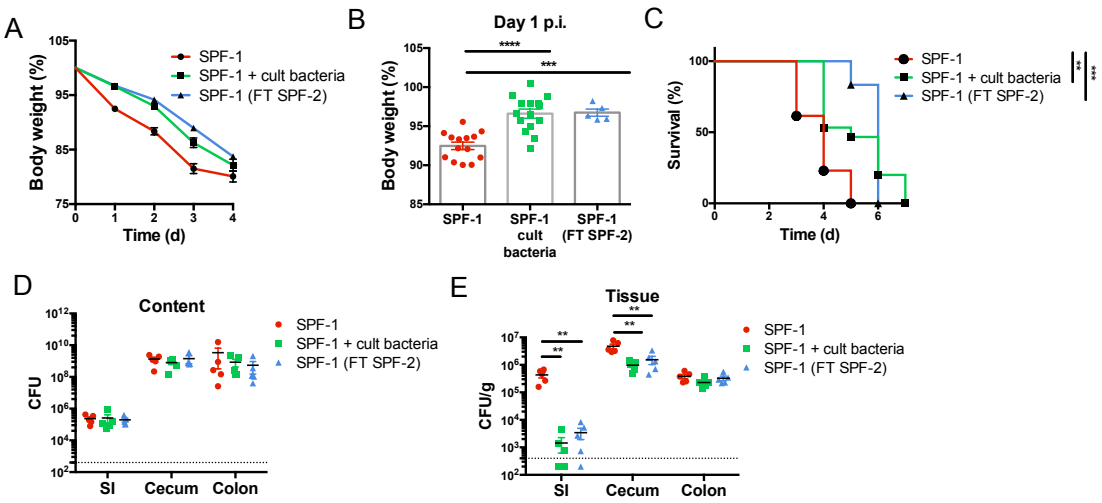

Figure 2.17 - Isolated bacteria derived from SPF-2 fecal content ameliorate *S.* Typhimurium infection. Isolated bacteria from SPF-2 mice were transferred to SPF-1 (SPF-1 + cult bacteria). As control whole SPF-2 community was transferred by fecal transplantation (SPF-1(FT SPF-2)). (A-C) Body weight was recorded during infection (A) and body weight of individual mice on day 1 p.i. is shown (B). Survival was measured during infection (C). (D-E) Salmonella infected mice were sacrificed 20 hours p.i. and number of *S.* Typhimurium CFUs was determined in luminal content (D) and tissue (E) of small intestine (SI), cecum and colon. Dashed line indicates the limit of detection. Results represent n=4-15 mice/group from one experiments as mean ± SEM. *P* values indicated represent a Mann-Whitney U test comparison of totals between groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

2.5 Discussion

It has been firmly established that the intestinal microbiota has a tremendous impact on susceptibility to enteric infection and the severity of intestinal inflammation (Buffie and Pamer, 2013). But only recently studies have started to reveal the identity of individual members of the complex gut communities that are responsible for protection of the host against gastrointestinal pathogens. Here, we dissected the influence of microbiota composition and specific members of the microbiome on the severity of disease induced by *S.* Typhimurium in a mouse model of acute gastrointestinal disease. While in mice *Salmonella* infection causes a disease resembling human typhoid fever without gastroenteritis, antibiotic pre-treatment enables colonization of the intestine by *Salmonella*, which subsequently leads to intestinal inflammation (Kaiser et al., 2012). This model has been widely used to study the contribution of bacterial virulence factors and host immune pathways to disease development and more recently to characterize the interplay between pathogen, microbiota and the mucosal immune system.

Specifically, it had been noted that mice from different conventional colonies, presumably with altered microbiota composition, differ in severity of intestinal pathology (Kaiser et al., 2012). Moreover, treatment with different antibiotics was found to cause distinct changes in the microbiome that were associated with varying susceptibility to *Salmonella* induced colitis despite comparable luminal colonization (Ferreira et al., 2011). We decided to follow up on these observations and took advantage of the striking differences observed in the microbiota of genetically highly related wild type mice obtained from different vendors, that we propose could serve as a valuable model representing the diverse microbiota compositions observed in humans. Indeed, large differences in weight loss and mortality that are surrogates for intestinal inflammation were readily detectable. To avoid any influence of genetic drifts and spontaneously acquired mutation in the investigated mouse lines, which have recently hampered researchers in several cases (Kayagaki et al., 2011; Vande Walle et al., 2016), we transferred the respective microbiota of susceptible and resistant mouse lines into genetically identical recipient mice. Infection experiments with such mice eventually confirmed our conclusion that

differences in microbiota composition were solely responsible for the altered disease severity. Strikingly, the altered disease severity was not associated with differences in luminal colonization, but rather with lowered *Salmonella* loads in the small intestinal and cecal tissues. This suggested that differences in microbiota composition were indirectly affecting severity of disease, potentially by modulating inflammatory responses and thus, *Salmonella* invasion and expansion in the host. At least two virulence strategies are involved in causing intestinal inflammation by *Salmonella*: The classical pathway that requires invasion of the intestinal epithelium enabled by virulence factors, which are encoded on *spi*-1, and the alternative pathway involves uptake and survival in phagocytic cells and requires virulence factors encoded on *spi*-2 (Kaiser 2011). We could rule out an involvement of the alternative pathway underscoring that specific commensals indirectly modulate invasion of epithelial cells or pathogen replication in non-phagocytic cells that trigger disease. The induced mucosal inflammation between the mouse lines was distinct as higher concentrations of the inflammatory marker IL-6 were detected in susceptible mice featuring higher *Salmonella* loads in the tissue. Amelioration of disease severity was associated with higher concentrations of IFNγ in the tissue. By using IFNγ deficient mice we demonstrated that IFNγ was essential for the modulatory effects of the protective microbiota (Figure 2.18). Enhancement of IFNγ expression by specific members of the protected microbiota might benefit the host via previously identified mechanisms such as controlling pathogen loads in the intestinal tissue and regulating mucin release by goblet cells (Godinez et al., 2008; Rhee et al., 2005; Songhet et al., 2011).

Much attention has recently focused on characterizing the influence of the microbiota and specific commensals on regulating development and recruitment of distinct subsets of immune cells including innate and adaptive lymphocytes (Honda and Littman, 2016; Klose and Artis, 2016). In order to identify in which cellular population IFNγ production is modulated in SPF-1 and SPF-2 mice, we used an IFNγ reporter mouse that allows identification of *in vivo* cytokine producing cells without further manipulations. As previously reported we detected IFNγ production in both $CD3^-$ (containing both classical

NK cells and ILCs) and $CD3^+$ cells (Dougan et al., 2011; Kupz et al., 2013; Spits et al., 2016). The frequency of IFNγ producing $CD3^+CD4^+$ T cells was increased in SPF-2 mice, whereas the abundance of other IFNγ producing subsets was not changed. These cells were negative for IL-17 and thus likely represent bona fide T_H1 cells. Moreover, we noted also enhanced frequencies of IL-17-producing T_H17 cells in the SI during infection. Induction of T_H17 cells by SFB contributes to the immune defense against the enteric pathogen *Citrobacter rodentium* (Ivanov et al., 2009). Moreover, SFB have been reported to induce IFNγ from $CD4^+$ T cells in SFB-monocolonized mice, consequently we considered the possibility that the presence of SFB in SPF-2 mice may be responsible for the observed phenotype (Gaboriau-Routhiau et al., 2009). But, colonization of SPF-1 mice with SFB did not confer protection and indicated that other commensals were responsible for enhancing IFNγ production from $CD4^+$ T cells (Figure 2.18).

In order to identify the bacteria responsible for amelioration of *Salmonella*-induced diseases we combined several approaches. First, 16S rRNA sequencing identified specific, but complex, signatures in the microbiota composition at all tested time points. Second, comparison of the phenotype and microbiome after pretreatment using streptomycin and ampicillin, antibiotics with distinct antimicrobial spectrum, demonstrated that both groups of SPF-2 mice were protected but each antibiotic treatment resulted in very distinct changes in the microbiome. Specifically, in the case of ampicillin the community of SPF-2 mice post-treatment resembled the community observed in ampicillin-treated SPF-1 mice, which were susceptible. Combining this observation with the notion that induction of T cell differentiation required longer periods, we therefore hypothesized that commensal bacteria, which were differentially abundant prior to antibiotic treatment, are required for the protection against *Salmonella* infection. We utilized an approach that had been used to perform highly parallelized isolation of intestinal bacteria and were able to short-term culture a restricted collection of eleven bacteria from five families that represented nine novel species as well as a *Lactobacillus intestinalis* and a *Bacteroides acidifaciens* isolate. Transfer of these bacteria into susceptible SPF-1 mice reduced tissue invasion by *Salmonella* and

delayed *Salmonella*-induced disease comparable to transfer of the total SPF-2 community. The protective effect of this restricted community is likely mediated via priming of the immune system rather than direct interaction with *Salmonella*. While transferred bacteria add up to around 37% of total 16S rRNA reads before antibiotic treatment, most of them are lost after streptomycin treatment and are further decreasing during *Salmonella* infection (< 3% of total 16S rRNA reads). Another recent study showed that a 33 member bacterial community derived from human isolates could reduce systemic *Salmonella* colonization independent of direct colonization resistance supporting our findings (Martz et al., 2015). The cultured bacteria, which were able to transfer the phenotype, included members of the families Prevotellaceae, Bacteroides, S24-7 (all belonging to the phylum Bacteroidetes), Lactobacillaceae and Lachnospiraceae (both belonging to the phylum Firmicutes) (Figure 2.18). While up to now, none of these bacteria have been causally linked to modulate severity of *Salmonella* infection or to regulate T_H1 responses, representatives from some of theses families have been correlated with less severe gastrointestinal infections in humans and mice. For instance, the abundance of the genus *Prevotella* demonstrated a negative correlation with potentially pathogenic *E. coli*/*Shigella* in a large survey study of small children from the developing world (Pop et al., 2014). Moreover, presence of *Prevotella copri*, a human-restricted member of the Prevotellaceae family, was enriched in human volunteers, which showed less severe diarrhea after voluntarily ingestion of enterotoxigenic *Escherichia coli* infection compared to volunteers with severe diarrhea (Pop et al., 2016). The same study also identified that the presence of *Bacteroides vulgatus* predicts resistance against diarrhea while other Bacteroides species were associated with severe diarrhea indicating that species and possibly even strain level differences contribute to resistance to enteric infection. The family S24-7 recently proposed as "*Candidatus homeothermaceae*" (Ormerod et al., 2016) or *Muribaculum* (Lagkouvardos et al., 2016) has been originally identified as group "Mouse Intestinal Bacteria" and found to be enriched in 129S1/SvImJ mice that were colonized by *Salmonella* but resistant to colitis (Ferreira et al., 2011). Additionally, this family is closely related to the Barnesiella family that has been strongly associated with protection against infection with

Vancomycin-resistant *Enterococcus faecalis* (Ubeda et al., 2013). However, the lack of available isolates has so far precluded functional studies that could be highly relevant due to its high abundance in the microbiota of many mouse lines and also some humans (Ormerod et al., 2016). Lachnospiraceae, previously known as *Clostridium* XIVa, are known butyrate-producing bacteria. It has been demonstrated that they induce regulatory $CD4^+$ T cells, but not T_H1 cells, in the colon (Atarashi et al., 2011). Currently we do not know if and which individual bacteria of the small community are responsible for the identified phenotype, but disentangling the possible interactions of the individual members of this community through combinatorial testing will be a challenging and laborious task as studies have highlighted that production of numerous metabolites might be dependent on interaction between bacteria (Faith et al., 2014).

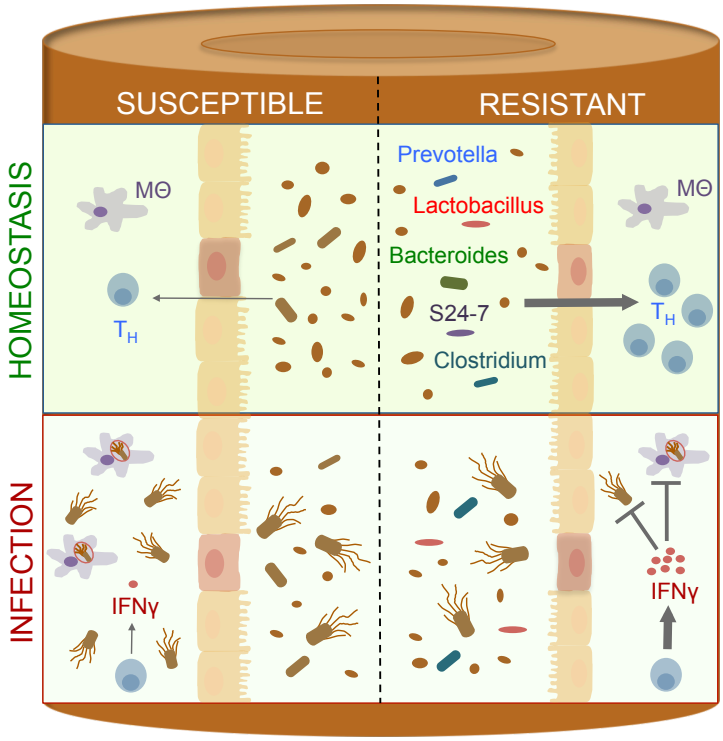

Figure 2.18 - Resident bacteria confer protection via IFNγ producing CD4+ T cells. Specific intestinal bacteria were identified to ameliorate acute *Salmonella* induced diarrhea, likely by priming the immune system. This is achieved by enhancing antibacterial IFNγ production by CD4+ T cells during infection preventing tissue invasion by the pathogen. Targeting this axis may benefit the development of novel probiotic interventions. IFN – Interferon, MΘ – macrophage, T_H – T helper cell

Our study has provided firm evidence that microbiota composition strongly influences severity of *Salmonella*-induced disease via a previously unknown modulation of mucosal IFNγ production. Former studies largely focused on identifying commensals that influence *Salmonella* colonization in the lumen, however, our experiments demonstrate that a restricted consortium of 11 bacteria is sufficient to ameliorate disease in susceptible mice by reducing pathogen colonization of the mucosal tissue. In conclusion, our study revealed a novel mechanism how the microbiota contributes to amelioration of *Salmonella* infection via microbiota-induced priming of a protective antibacterial response by the mucosal immune system.

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Abbreviations

ANOVA	Analysis of variance
ASF	Altered Schaedler flora
BCA	Bicinchoninic acid
BHI	Brain heart infusion
BSL	Baseline
CD	Cluster of differentiation
CFU	Colony forming units
Coh	Cohoused
Duo	Duodenum
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylendiamintetraacetat acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FoxP3	Forkhead box protein 3
FT	Fecal transplant
GI	Gastrointestinal tract
GF	Germ-free
HBSS	Hank’s buffered salt solution
HCL	Hydrogen chloride
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
Jej	Jejunum
Lac	Lactose-fermenting
LB	Lysogeny broth
LDA	Linear discriminant analysis
LEfSe	LDA effect size
MΘ	Macrophage
MPN	Most probable number
MLN	Mesenteric lymph node
NaCl	Sodium chloride
NK	Natural killer cell

NTS	Non-typhoidal *Salmonella*
o.g.	Oral gavage
OTU	Operational taxonomic level
PBS	Phosphate buffered saline
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
p.i.	Post infection
pIL	Proximal ileum
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SFB	Segmented filamentous bacteria
SI	Small intestine
SPF	Specific pathogen free
SPI	*Salmonella* pathogenicity island
S.T.	*Salmonella* Typhimurium
Strep	Streptomycin
T3SS	Type III secretion system
TAE	Tris acetat EDTA buffer
TCR	T cell receptor
TE	Tris EDTA
T_H	T helper lymphocyte
tIl	Terminales ileum
Tris	Tris(hydroxymethyl)-aminomethan
WT	Wild type

3 CHAPTER

Identifying microbial signatures associated with reduced susceptibility to *Citrobacter rodentium* infection

3.1 Summary

Infectious diarrhea, induced by foodborne pathogens, is a significant health problem worldwide. Upon oral uptake, pathogens encounter in the gastrointestinal tract a diverse community of commensal bacteria, collectively termed the microbiota. An undisturbed microbiota greatly reduces the chances of a successful colonization by the invading enteric pathogens. But, an imbalanced community may lead to dysregulation of the intestinal homeostasis and, in turn, facilitate colonization and inflammation of enteric pathogens. Specific interactions between distinct groups of commensals and pathogens are hypothesized to determine an individual's susceptibility to infection. However, the contributions of individual members of the microbiota are largely unknown.

Here, we demonstrate that C57BL/6N mouse lines from different breeding facilities display highly varying susceptibility to infection with the pathogen *Citrobacter rodentium*, a mouse model for human enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC). Using 16S rRNA sequencing, we identified a microbial signature associated with reduced pathogenic colonization. By using cohousing experiments with susceptible and resistant mouse lines, bacteria of the family *Lachnospiraceae* were highly linked to amelioration to *C. rodentium* induced disease. Identifying intestinal bacteria, which enhance protection against enteric infections, is crucial for the development of novel mucosal therapies.

3.2 Introduction

The gastrointestinal tract (GI) is colonized by a complex bacterial community, which has a large impact on the host immune system playing an essential role in health and disease. One major task of the microbiota is to protect the host against invading pathogens via a process called colonization resistance (Buffie and Pamer, 2013). Colonization resistance is realized via direct microbiota-pathogen interactions (e.g. competition for essential nutrients, production of toxic compounds, occupancy of niches), but also indirectly via immune-mediated mechanisms through the induction of immune cells that target enteric pathogens. Environmental factors such as antibiotic treatment affect strongly the composition of the intestinal microbiota, leading to severe dysregulation of the intestinal homeostasis. For instance, altered gut composition induced by antibiotic treatment can influences colonization of invading enteric pathogens (Bohnhoff and Miller, 1962; Sekirov et al., 2008).
Moreover, composition of the microbiota greatly varies among individuals, possibly explaining different susceptibilities to enteric infections (Human Microbiome Project Consortium, 2012).
Infectious diarrhea, especially caused by enteric pathogens, is a major health problem worldwide, contributing significantly to morbidity and mortality (WHO, 2013). In particular, the foodborne pathogens enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) can cause a severe infection characterized by GI inflammation and in some cases life-threatening diarrhea (Collins et al., 2014).
The mouse-restricted pathogen *C. rodentium* is closely related to the clinically important human pathogens EHEC and EPEC, also belonging to the pathogenic family of A/E lesion-forming bacteria (Petty et al., 2010). Highly similar to EHEC and EPEC, attaching *C. rodentium* efface the microvilli and induce cytoskeletal changes such as accumulation of polymerized actin, forming distinct pedestal-like structure beneath the site of attachment. Genes important for formation of A/E lesions are encompassed within a 35 kb pathogenicity island known as the locus of enterocyte effacement (LEE), encoding the type III secretion system (T3SS) (Deng et al., 2004). Via the T3SS effector proteins are translocated into the host cell cytoplasm. One

essential and well studied virulence factor important for inducing inflammation of *C. rodentium* is the translocated intimin receptor (Tir), similar to Tir expressed in EHEC and EPEC (Deng et al., 2003). During the attachment to gut enterocytes, *C. rodentium* does not bind to a host receptor, but instead translocate its own receptor Tir into the cytoplasm of host cell. After translocation Tir is displayed on the surface of enterocytes, to which the outer-membrane bacterial protein intimin binds, triggering host signaling events (Deng et al., 2003; Kenny et al., 1997). Colonization of *C. rodentium* leads to infectious colitis characterized histologically by immune cell infiltration, intestinal crypt elongation and depletion of goblet cells (Bhinder et al., 2013). Since EHEC and EPEC are mainly human pathogens, which induce only modest pathogenicity in mice, *C. rodentium* is a valuable and frequently used mouse model for these infections (Mundy et al., 2005).

In last years, several studies highlighted the large effect of the microbiota composition on the intestinal colonization of *C. rodentium*. For instance, antibiotic treatment with metronidazole has been shown to disrupt the microbiota composition, increasing susceptibility to *C. rodentium* infection due to an altered goblet cell function (Wlodarska et al., 2011). Strikingly, in the same study antibiotic treatment with streptomycin has been shown to alter the composition of the microbiota, but do not affect severity of infection (Wlodarska et al., 2011). Therefore, this study implicated that specific, still unknown bacteria are responsible for protection rather than the decrease in diversity, which is induced upon antibiotic treatment. Additionally, successful fecal transplantation from resistant to susceptible mice to *C. rodentium*-induced colitis suggests the importance of the microbiota to protect the host against enteric pathogens (Willing et al., 2011). Interestingly, fecal transplantation increased interleukin (IL)-22-mediated defense coupled with augmented antimicrobial peptides regenerating islet-derived 3 (Reg3)γ and Reg3β, which decreased susceptibility to infection (Willing et al., 2011). This study indicated how yet unknown bacteria promote resistance indirectly, by enhancing immune-mediated protection to *C. rodentium* infection. In addition, changing the diet of the host has been shown to promote higher abundance of Clostridia species, which were associated to influence the epithelial barrier, therefore, indirectly affecting luminal colonization of C. rodentium (Wlodarska

et al., 2015). Also direct mechanisms of commensal bacteria have been identified to target *C. rodentium* colonization. During infection, expansion of commensal *E. coli* inhibits *C. rodentium* colonization via competing for monosaccharides as nutrient (Kamada et al., 2012).

Although, it is known that the intestinal microbiota affects the susceptibility to infections of *C. rodentium*, research is still in its infancy and little is know which specific commensal bacteria and which pathogen-microbiota interactions contribute to protection against colonization of *C. rodentium*.

In this study, we could demonstrate that isogenic C57BL/6N mice from different breeding facilities feature distinct microbiota compositions, which also differ in their susceptibility to *C. rodentium* infection. Correlation analysis including cohousing experiments with susceptible and resistant mice could reveal that two bacteria from the family Lachnospiraceae are associated with lower luminal colonization of *C. rodentium*. Detailed understanding how commensal bacteria interact with the pathogen and the host to confer resistance is important for the development of therapies to manipulate the microbiota against infections.

3.3 Methods

3.3.1 Mice

C57BL/6N SPF-1 mice were purchased from NCI and maintained (including breeding and housing) at the animal facilities of the Helmholtz Centre for Infection Research (HZI) under enhanced specific pathogen-free (SPF) conditions. C57BL/6N SPF-2, SPF-3, SPF-4, SPF-6 and SPF-7 mice were purchased from different vendors and housed under enhanced SPF conditions at the HZI for at least two weeks before the start of the experiment: Charles River (SPF-2 and SPF-3), Harlan (SPF-4), Janvier (SPF-6), and Taconic (SPF-7). SPF-5 mice were generated, bred and housed under enhanced SPF conditions at the HZI (Stehr et al., 2009). Germ-free C57BL/6NTac mice were bred in isolators (Getinge) in the germ-free facility at the HZI.

Animals used in experiments were gender and age matched. Mice with an age of 8-12 weeks were used. Mice were provided with sterilized food and water ad libitum. Mice were kept under strict 12-hour light cycle (lights on at 7:00 am and off at 7:00 pm) and housed in groups of up to 6 mice per cage. All mice were euthanized by asphyxiation with CO_2 and cervical dislocation. Both female and male mice were used in experiments. All animal experiments have been performed in agreement with the local government of Lower Saxony, Germany (approved permission No. 33.4-42502-04-14/1415).

3.3.2 Microbiota manipulation

For cohousing experiments with conventional raised mice, age- and gender-matched C57BL/6N mice were housed together in cages at 1:1 ratios for at least four weeks before infection experiments. For cohousing experiments with germ-free mice, mice were cohoused at 1:1 ratio in specific ventilated isocages equipped with individual HEPA filters (Tecniplast).

3.3.3 *C. rodentium* infection

Bioluminescence expressing *C. rodentium* strain ICC180 was used for all infection experiments (Wiles et al., 2004). *C. rodentium* inoculums were prepared by culturing bacteria overnight at 37°C in LB broth with 50 µg/ml

kanamycin. Subsequently, the culture was diluted 1:100 in fresh medium, and subcultured for 4 hours at 37°C in LB broth. Bacteria were washed twice in ice-cold phosphate-buffered saline (PBS). Mice were orally inoculated with 10^7 CFU of *C. rodentium* diluted in 200 µl PBS. Weight of the mice was monitored and feces were collected every second day after infection for measuring pathogen burden and 16S rRNA sequencing.

3.3.4 Quantification of fecal *C. rodentium* colonization

Fresh fecal samples were collected and weight was recorded. Subsequently, fecal samples were homogenized in 1 ml LB media by bead-beating with 1mm zirconia/silica beads. Two samples were homogenized for 25 sec using a Mini-Beadbeater-96 (BioSpec). To determine CFUs, serial dilutions of homogenized samples were plated on LB and MacConkey plates, if necessary 50 µg/ml Kanamycin was added to media. Plates were cultured at 37°C for 1 day before counting. CFUs of *C. rodentium* were calculated after normalization to the weight of feces.

3.3.5 DNA isolation and library preparation

DNA was extracted using a phenol-chloroform method as describe previously (Turnbaugh et al., 2009). Briefly, extraction buffer (200 mM Tris, 20 mM EDTA, 200 mM NaCl, pH 8.0), 200µl of 20% SDS and 500 µl of phenol:chloroform:isoamyl alcohol (24:24:1) was added to frozen samples. To lyse bacterial cells, mechanical disruption method was chosen using 0.1-mm diameter zirconia/silica beads and Mini-BeadBeater-96 (BioSpec). For lysis, samples were homogenized twice for 2 min. After centrifugation, aqueous phase was passed for another phenol:chloroform:isoamyl alcohol extraction. Furthermore, 0.1 volume of 3 M sodium acetate and an equal volume of ice-cold isopropanol were added to precipitate DNA. Samples were incubated at -20°C for at least 1 hour and then centrifuged at 4°C at maximum speed for 20 min. Resultant pellet was washed with 75% ethanol and dried by using speed vacuum for 15 min. Crude DNA pellets were resuspended in TE buffer with 100 µg/ml RNAse and subsequently, purified by column purification (BioBasic). To perform 16S rRNA gene amplification of V4 16S rRNA region, crude DNA was normalized to 25 ng/µl. A unique 12-base Golary barcode to

each sample was incorporated via specific primers for identification (Caporaso et al., 2011). PCR was performed in triplicates and pooled afterwards. Genomic DNA was quantified by using PicoGreen dsDNA quantitation kit (Invitrogen) and normalized to 10 nM DNA. Samples were pooled and quantified by using KAPA Library Quantify Kit SYBR (Kapa Biosystems).

3.3.6 16S rRNA analysis

Amplification of the V4 region (F515/R806) of the 16S rRNA gene was performed according to previously described protocols (Caporaso et al., 2011). Samples were sequenced on an Illuminia MiSeq platform via 250 bp paired-end sequencing. Sequences were filtered for low quality reads and binned based on sample-specific barcodes using QIIME v1.8.0 (Caporaso et al., 2010). Quality filtering was set up to -q 30, minimum read length 200 bp and minimum number of sequences per sample = 1000. Reads were clustered into 97% ID OTUs using open-reference OTU picking with UCLUST (Edgar, 2010), followed by abundance filtering (OTUs cluster > 0.5%) and taxonomic classification using the RDP Classifier executed at 80% bootstrap confidence cut off (Wang et al., 2007). Sequences without matching reference dataset, were grouped as *de novo* using UCLUST. Phylogenetic relationships between OTUs are determined using FastTree to the PyNAST alignment (Price et al., 2010). The OTU absolute abundance table and mapping file are used for statistical analyses and data visualization in the R statistical programming environment package phyloseq (McMurdie and Holmes, 2013).
The phylogenetic tree was created using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) is shown next to the branches (Zharkikh and Li, 1995). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

3.3.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism v6.0. Differences were analyzed by Student’s t test and one-way ANOVA.

P values indicated were calculated by a non-parametric Mann-Whitney U test or Kruskal-Wallis test comparison of totals between groups. Linear discriminant analysis (LDA) effect size (LEfSe) method was used to identify bacterial OTUs that explained differences between microbiota compositions (Segata et al., 2011). OTUs with Kruskal-Wallis test <0.05 and LDA scores >3.0 were considered for analysis.

P values lower than 0.05 were considered as significant: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

3.4 Results

3.4.1 Isogenic mouse lines from different breeding facilities reveal distinct microbiota compositions

In order to identify bacteria linked to resistance against *C. rodentium* infection, we evaluated isogenic mouse lines regarding potential differences in microbiota composition that could reflect the diverse microbiome in humans. To this end, we first screened the microbiota of age- and gender-matched C57BL/6N specific pathogen free mice (SPF) from different breeding facilities and barriers (SPF-1 – SPF-7). Mice were housed in our vivarium for at least 10 days to adjust for housing conditions (diet, water, bedding). To analyze the composition of the microbiota, 16S rRNA analysis of the fecal microbiota was performed. Analysis of β-diversity revealed differences in the microbiota of each breeding facility (Figure 3.1 A).

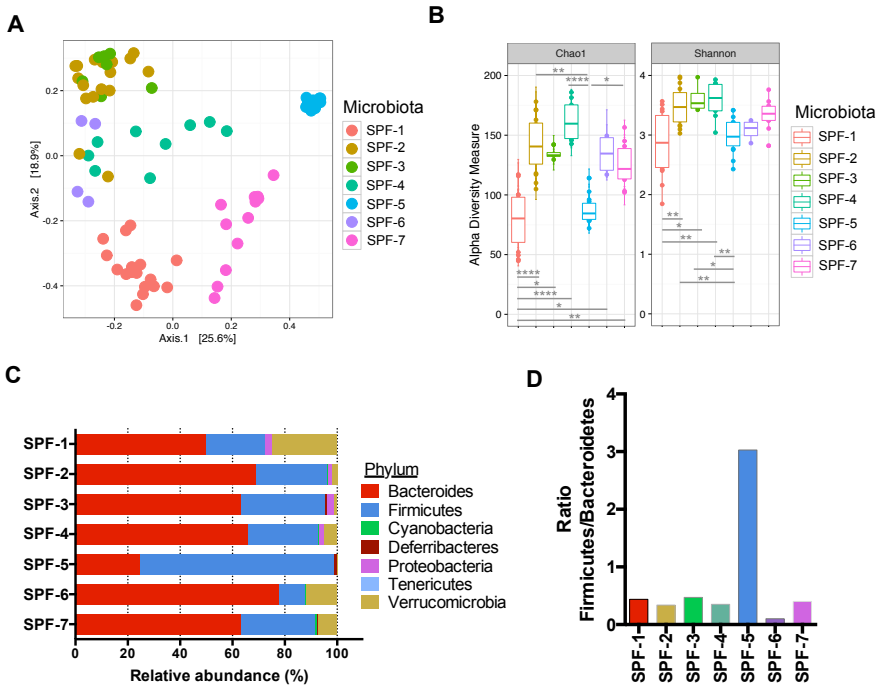

Figure 3.1 - **Isogenic mouse lines feature distinct microbiota compositions.** Fecal microbiota of different specific pathogen free (SPF) mouse lines including SPF-1 (n=17), SPF-2 (n=18), SPF-3 (n=7), SPF-4 (n=9), SPF-5 (n=15), SPF-6 (n=4) and SPF-7 (n=12) were evaluated using 16S rRNA sequencing. (A) β-diversity was analyzed using Bray-Curtis dissimilarity matrix and PCoA plot. (B) α-diversity was determined using Chao1 and Shannon index. (C) Relative abundance at phylum level for each group is shown. (D) Ratio of taxonomic order Firmicutes and Bacteroidetes for each group. Representative data derived from one experiment (SPF-4, SPF-6) or are pooled from at least two different experiments (SPF-1, SPF-2, SPF-3, SPF-5, SPF-7). P values indicated represent a nonparametric Kruskal-Wallis test with multiple comparisons (one-way ANOVA). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Samples clustered largely according to mouse line. Specifically, mouse lines from different breeding facilities harbored a distinct composition, whereas the mouse lines from the same vendor but different barriers (SPF-2 and SPF-3) had a similar microbial composition.

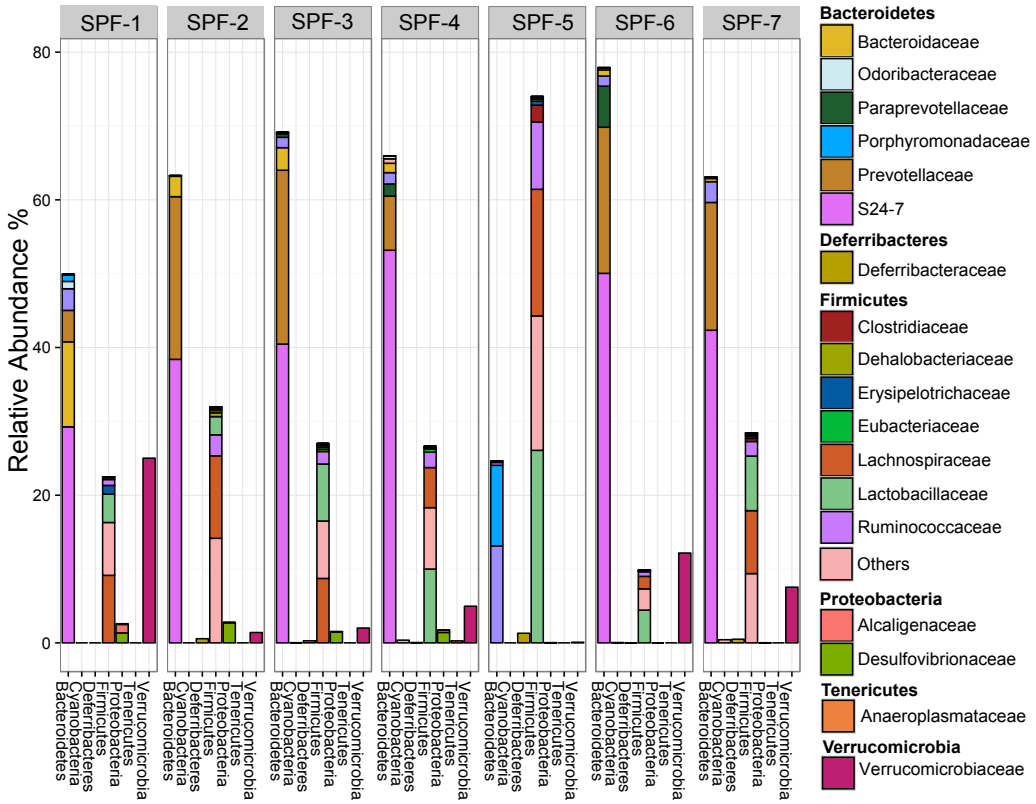

Figure 3.2 - Mice from different vendors reveal differences in their microbiota composition at taxonomical level of family. Fecal microbiota of different specific pathogen free (SPF) mouse lines including SPF-1 (n=17), SPF-2 (n=18), SPF-3 (n=7), SPF-4 (n=9), SPF-5 (n=15), SPF-6 (n=4) and SPF-7 (n=12) was analyzed using 16S rRNA sequencing. Relative abundances of bacterial families are shown and grouped according to their phylum. Bars represent the mean of all mice within the group. Representative data are from one experiment (SPF-4, SPF-6) or pooled from at least two different experiments (SPF-1, SPF-2, SPF-3, SPF-5, SPF-7).

Next, α-diversity (species diversity within the community of each sample) was analyzed by using Chao1 (species richness) and Shannon indices (species richness combined with abundance) between the isogenic mouse lines (Figure 3.1 B). All mouse lines harbored a complex microbiota, but the microbial diversity varied between the groups. The microbiota of SPF-1 and SPF-4 mice showed a significantly lower α-diversity than the microbiota of SPF-2, SPF-3, SPF-5, SPF-6 and SPF-7 mice.

In addition, microbial composition was analyzed at different taxonomic levels (phylum, family, genus/OTU). Analysis of the relative abundances of bacterial phyla identified differences already at this level affecting the phyla Bacteroides, Firmicutes, Proteobacteria and Verrucomicrobia (Figure 3.1 C). In particular, ratio of Firmicutes to Bacteroides level is highly increased in SPF-5 mice, whereas the other microbiota settings reveal a higher abundance of Bacteroidetes (Figure 3.1 D).

Analysis of lower taxonomical level (family and OTU level) discovered large microbial differences in the bacterial families of Bacteroidaceae (i.e. high in SPF-1; absent in SPF-5 and SPF-7), Prevotellaceae (i.e. high abundant in SPF2 and SPF-3; absent in SPF-5), S24-7 (i.e. high abundant in SPF-6; absent in SPF-5), Desulfovibrionaceae (i.e. high in SPF2 and SPF-3; absent in SPF-5, SPF-6 and SPF-7), Verrucomicrobiaceae (i.e. high abundant in SPF-1; absent in SPF-5) (Figure 3.2 and 3.3).

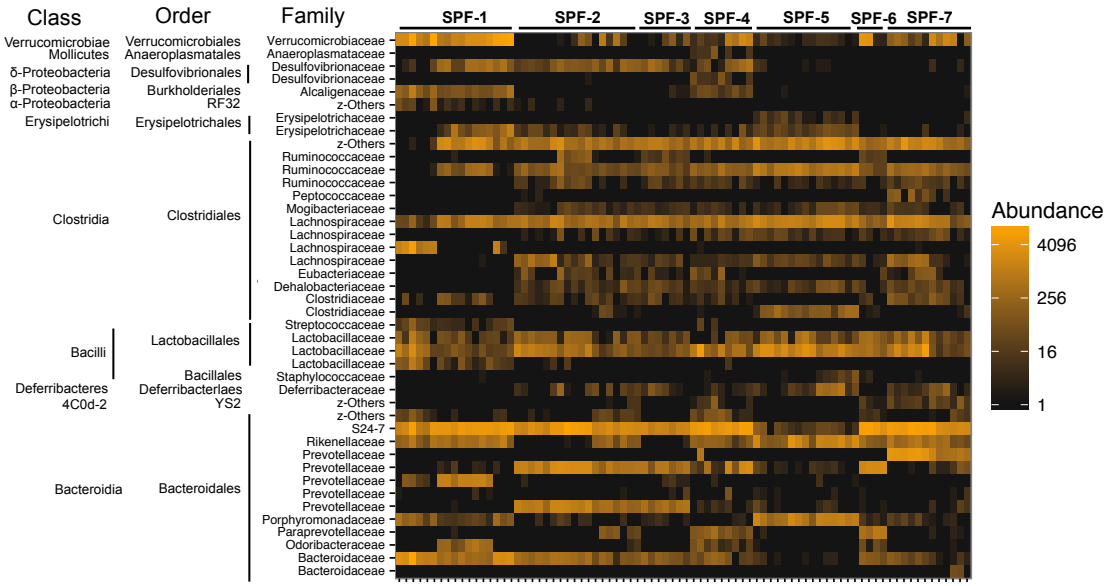

Figure 3.3 - Mice from different vendors display distinct microbiota composition at OTU level. Fecal microbiota of different specific pathogen free (SPF) mouse lines including SPF-1 (n=17), SPF-2 (n=18), SPF-3 (n=7), SPF-4 (n=9), SPF-5 (n=15), SPF-6 (n=4) and SPF-7 (n=12) was analyzed using 16S rRNA sequencing. Heatmap of relative abundances of bacterial OTUs (≥97% sequence similarity, >0.05% relative abundance) is sorted by family, class and order as well as mouse line. Each vertical bar represents the microbiota of an individual mouse. Representative data are from one experiment (SPF-4, SPF-6) or pooled from at least two different experiments (SPF-1, SPF-2, SPF-3, SPF-5, SPF-7).

In summary, by analyzing mice from different breeding facilities, we identified unique microbial signatures in the fecal microbiota of examined isogenic mouse lines. Differences were observed at all taxonomical level from phylum

to OTU level. Notably, mice derived from different barriers, but same breeding facility, revealed higher similarity in their microbial signature.

3.4.2 Isogenic mouse lines feature different susceptibilities to *C. rodentium* infection

Since isogenic mice from different breeding facilities harbor distinct microbial communities in their intestine, we hypothesized that they display distinct disease progressions when infected with *C. rodentium*. In order to test this hypothesis, age- and gender-matched SPF-1 – SPF-7 mice were infected with 10^7 CFU *C. rodentium* by oral gavage (Figure 3.4 A).

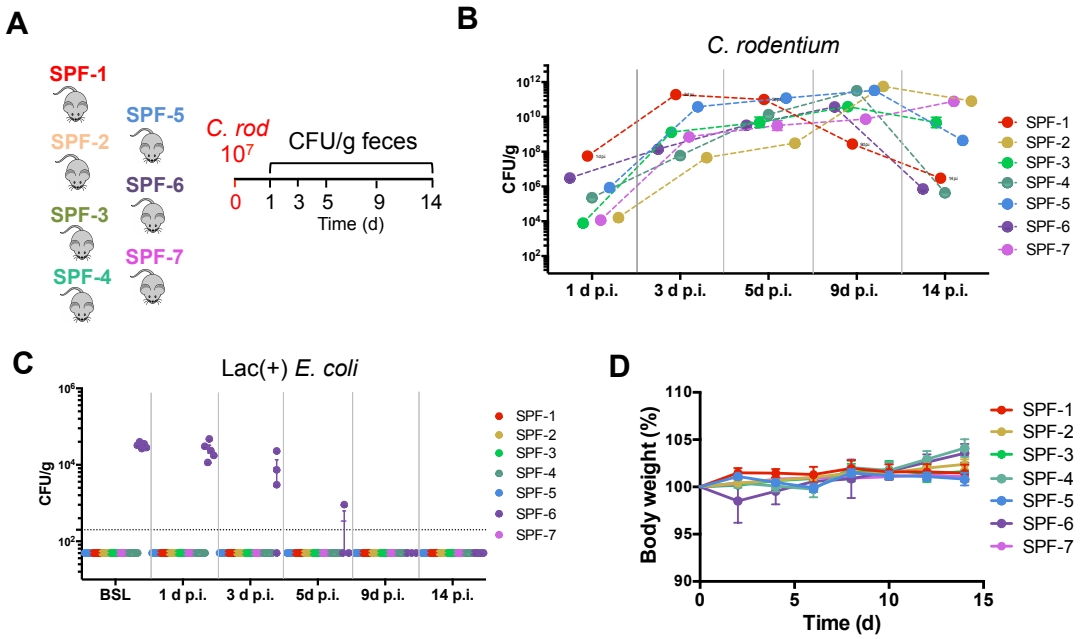

Figure 3.4 - Isogenic mouse lines differ in their susceptibility to *C. rodentium* infection. (A) Mice with different microbiota settings (SPF-1-SPF-7) were infected orally with 10^7 CFU *Citrobacter rodentium* (*C. rod*). (B) CFU of *C. rod* was determined in the feces of each mouse line at baseline before infection (BSL) and day 1, 3, 5, 9 and 14 post infection (p.i.). (C) Fecal samples were cultured on MacConkey agar during infection. Lactose-fermenting (Lac+) colonies represent *Escherichia coli.* (D) Body weight was recorded during the course of infection. Dashed lines indicate the limit of detection. Results represent n=4-18 mice/group as mean ± SEM from one experiment (SPF-4, SPF-6) or pooled from at least two different experiments (SPF-1, SPF-2, SPF-3, SPF-5, SPF-7).

Colonization of *C. rodentium* in feces was monitored longitudinally during infection and kinetic of *C. rodentium* colonization was noted to be significantly different between mouse lines (Figure 3.4 B and 3.5).

In the first days after infection (day 1 and 3 p.i.), SPF-1 mice showed the highest pathogen burden in stool, whereas SPF-2, SPF-3, and SPF-7 mice had the lowest colonization. In contrast, SPF-4, SPF-5, and SPF-6 displayed an intermediate susceptibility at day 1 p.i (Figure 3.5). Clearance of of *C. rodentium* is delayed in SPF-2 mice, which showed a significantly higher colonization as seen in SPF-1 and SPF-4 mice at later analyzed time points (day 14 p.i.).

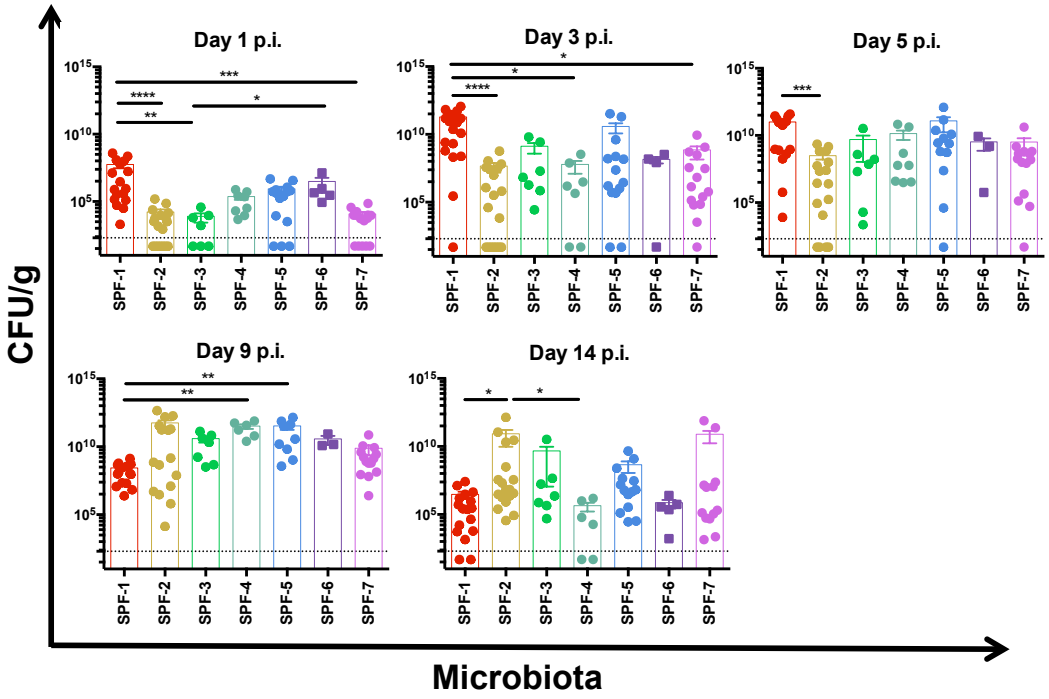

Figure 3.5 - Colonization kinetics during *C. rodentium* differs in isogenic mouse lines. *C. rodentium* CFUs in feces of individual mice of each mouse line are displayed on day 1, 3, 5, 9 and 14 post infection (p.i.). Dashed lines indicate the limit of detection. Results represent n=4-18 mice/group as mean ± SEM from one experiment (SPF-4, SPF-6) or pooled from at least two different experiments (SPF-1, SPF-2, SPF-3, SPF-5, SPF-7). *P* values indicated represent a nonparametric Kruskal-Wallis test with multiple comparisons (one-way ANOVA). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

It has been reported that gut inflammation favors the outgrowth of Enterobacteriaceae from within the host intrinsic commensal microbiota and that they may act as competitors of invading pathogens (e.g. commensal *E. coli* during *S.* Typhimurium infection) (Lupp et al., 2007; Stecher et al., 2007). Therefore, we quantified the level of Enterobacteriaceae before and during infection by culturing stool on MacConkey agar plates (selective for Gram-negative enteric bacteria). Lactose-utilizing (Lac+) colonies were detected at low numbers only in SPF-6 mice, but disappeared during infection (Figure 3.4

C). Lac+ colonies were isolated and their 16S rRNA gene was sequenced using Sanger sequencing identifying them as *E. coli*. In addition, *C. rodentium* is a delayed lactose fermenter and colonies could be distinguished by the appearance of a red center and colorless margin (Fox, 2007). CFUs of *C. rodentium* plated on MacConkey are identically with CFUs plated on LB agar added with kanamycin displayed in Figure 3.4 B (data not shown).

SPF-6 is the only mouse line, which harbor other Lac+ colonies than *C. rodentium*, identified as *E. coli*. Differently than reported during *Salmonella* infection (Stecher et al., 2013), enterobacterial bloom of *E. coli* was absent and *E. coli* disappeared in SPF-6 mice in the course of *C. rodentium* infection. Therefore, it is likely that *E. coli* is indispensable for resistance against *C. rodentium* infection.

In order to monitor severity of gastroenteritis, body weight was measured during infection. Severe body weight loss during disease was absent in all mouse lines (< 5% of weight loss). Moreover, no significantly differences in weight loss between mouse lines was observed (Figure 3.4 D).

Changes in the intestinal microbiota were analyzed by 16S rRNA analysis at different time points (before infection, day 1, 3, 5, 9, 14 p.i.) (Figure 3.6 A-E). Due to larger samples number per group, mouse lines with higher (SPF-1) and lower susceptibility (SPF-2, SPF-3, SPF-7) were selected.

In order to identify the microbial dynamic during infection, changes in the fecal microbiota was analyzed at different time points of the infection (before infection, day 1, 3, 5, 9 and 14 p.i.). As represented in a PCoA plot, microbial changes during different phases of infection are minor compared to the differences observed between mouse lines (Figure 3.6 A). In addition, relative abundances of bacterial families were examined for each microbiota setting during infection. At early time points of infection, microbial composition was relatively stable, whereas during course of disease differences in the bacterial abundances could be observed (Figure 3.6 B-E). In particular, SPF-7 mice displayed a high microbial dynamic, i.e. higher abundance of unclassified bacteria (indicated as “Others”) as well bacteria of the family Lachnospiraceae and Ruminococcaceae than before infection (Figure 3.6 E).

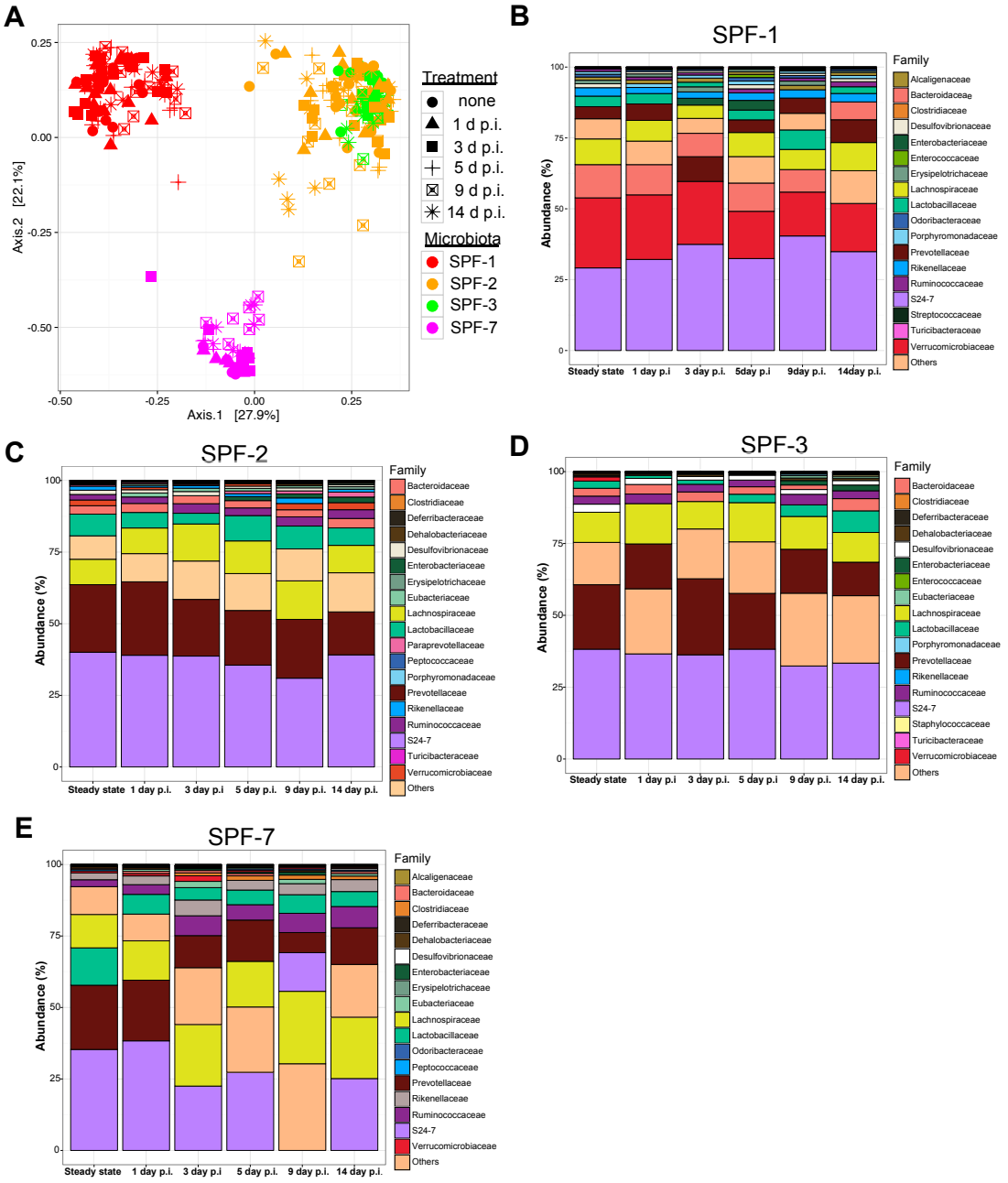

Figure 3.6 - Intestinal microbiota during different phases of *C. rodentium* infection. SPF-1 (n=17), SPF-2 (n=18), SPF-3 (n=7) and SPF-7 (n=12) were infected orally with *C. rodentium* and fecal microbiota was analyzed using 16S rRNA sequencing before infection, day 1, 3, 5, 9 and 14 post infection (p.i.). (A) β-diversity was analyzed using Bray-Curtis dissimilarity matrix and PCoA plot. (B-E) Relative abundances of bacterial families are shown before and during *C. rodentium* infection for each group, SPF-1 (B), SPF-2 (C), SPF-3 (D) and SPF-7 (E). Bars represent the mean of all mice within the group. Data are pooled from at least two different experiments.

In contrast, only small changes in the bacterial community during infection were observed in SPF-2 mice (Figure 3.6 C). Changes of the microbiota in SPF-1 and SPF-3 mice were low during infection and could be explained by altered bacterial abundances (Figure 3.6 A, 3.6 C). Besides in SPF-7 mice,

the microbiota of SPF-1, SPF-2 and SPF-3 mice do not show a significant outgrowth of a bacterial family, which could explain the resistance or susceptible phenotype.

To exclude that differences in the genotype between C57BL6/N sub-lines are responsible for the observed phenotype, we cohoused SPF-1 and SPF-2 mice with germ-free C57BL6/NTac mice for at least four weeks (Figure 3.7 A).

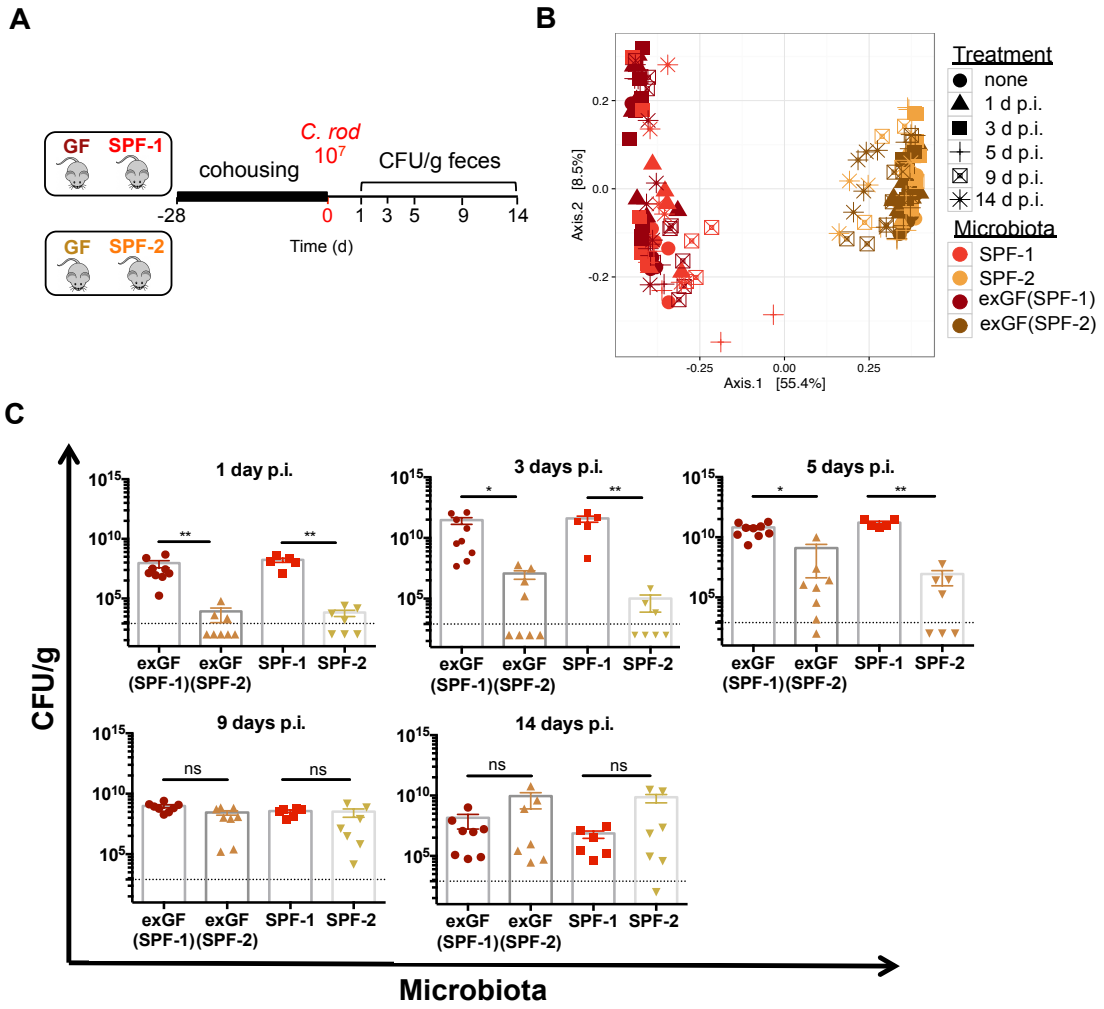

Figure 3.7 - Differences in disease severity are microbiota-dependent. (A) C57BL6/N germ-free mice (GF) were cohoused with SPF-1 and SPF-2 mice for 4 weeks and infected with *C. rodentium* (*C. rod*). (B) Fecal microbiota was analyzed using 16S rRNA sequencing before infection, day 1, 3, 5, 9 and 14 post infection (p.i.). β-diversity was analyzed using Bray-Curtis dissimilarity matrix and PCoA plot. (C) *C. rodentium* CFUs in feces of individual mice of each mouse line are displayed on day 1, 3, 5, 9 and 14 p.i. Dashed lines indicate the limit of detection. Results represent one independent experiment with n=5-9 mice/group as mean ± SEM. *P* values indicated represent a Mann-Whitney U test comparison between group exGF(SPF-1) and exGF(SPF-2) as well as SPF-1 and SPF-2. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Analyzing the microbiota by 16S rRNA sequencing after cohousing confirmed that recipient formerly GF mice feature the same microbiota composition in feces as respective donor mice (SPF-1, SPF-2) (Figure 3.7 B). Strikingly,

recipient GF mice show a similar degree of *C. rodentium* colonization as the respective SPF-1 or SPF-2 donor mice (Figure 3.7 C). The results show that the observed phenotype fully depends on the microbiota composition.
In summary, the results strongly suggest that isogenic C57BL/N mice differ in their kinetics of *C. rodentium* colonization and clearance. Cohousing experiments with germ-free mice indicate that colonization kinetics were truly influenced by the microbiota composition.

3.4.3 In isogenic mouse lines distinct bacteria are linked to resistance and susceptibility to *C. rodentium* infection

We hypothesized that the presence of specific bacteria is linked to higher or lower colonization at early time points of infection (resistant/susceptible). To identify potentially microbial biomarkers correlated with lower and higher *C. rodentium* colonization, the linear discriminant analysis (LDA) effect size (LEfSe) method, coupled with standard tests for statistical significance was used (Segata et al., 2011). The LEfSe algorithm contains different steps to detect differentially abundant bacteria. First, non-parametric factorial Kruskal-Wallis sum-rank test was implemented to identify bacteria with significant differential abundance with respect to class of interest (susceptibility). Subsequently, biologically consistency was determined among subclasses (mice from different breeding facilities) using the unpaired Wilcoxon rank-sum test. Afterwards, effect size of each differentially abundant bacterium was evaluated, ranked according to the effect size and displayed in a histogram. Analysis was focused on the microbiota composition at steady state in SPF-1 mice with the highest susceptibility and compared with SPF-2, SPF-3 and SPF-7 mice, showing the lowest susceptibility to *C. rodentium* at early time points. The most enriched bacterial taxa in susceptible mice belong to the family: Verrucomicrobiaceae, Bacteroidaceae and Alcaligenaceae (Figure 3.8 A-B). Specifically, bacteria of the family Bacteroidaceae are lower abundant in all microbiota settings linked to resistance (Figure 3.9 A). Alcaligenaceae are absent in microbiota settings linked to resistance (SPF-2, SPF-3, SPF-7) and only detectable in most of the SPF-1 mice (Figure 3.9 B).

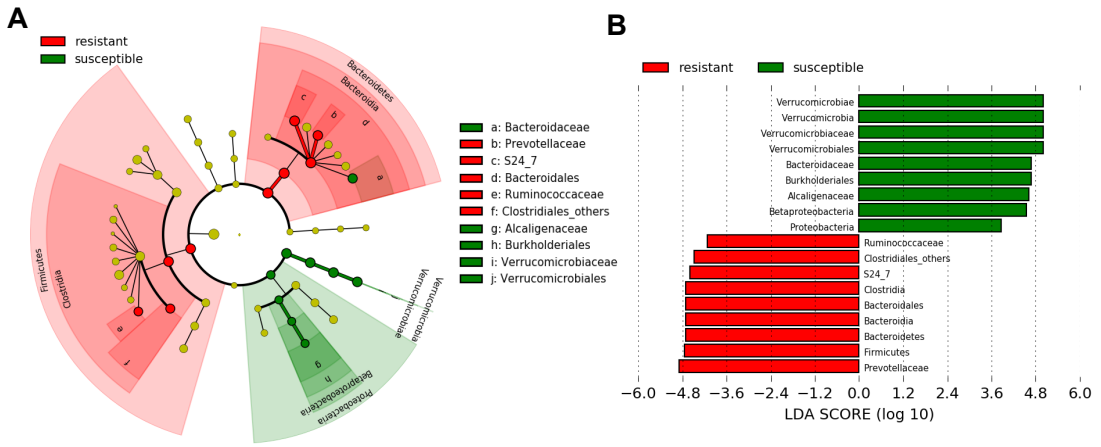

Figure 3.8 - Microbial signatures associated with reduced disease susceptibility of *C. rodentium*. Statistically significant bacterial differences in the fecal microbiota between susceptible (SPF-1) and resistant (SPF-2, SPF-3 and SPF-7) mice before treatment was analyzed at family level using LEfSe (Kruskal-Wallis and pairwise Wilcoxon test $p<0.05$, LDA >3.0). Data are displayed as cladogramm (A) and bars (B) ranking them accordingly to the effect size and associating them to their degree of susceptibility. Data are pooled from at least two different experiments with n=7-18 mice/group.

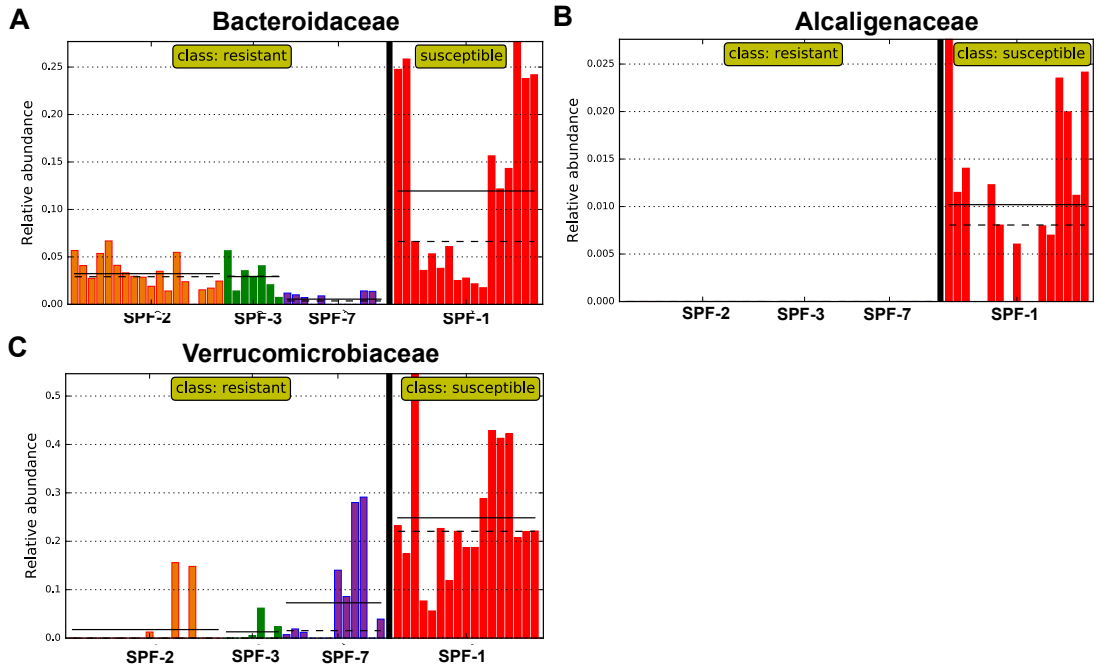

Figure 3.9 - Individual biomarkers associated with higher susceptibility. Statistically significant differences of microbial signatures linked to a higher degree of susceptibility are displayed of individual mice (SPF-1, SPF-2, SPF-3, SPF-7) on family level before infection: (A) Bacteroidaceae, (B) Alcaligenaceae, (C) Verrucomicobiaceae. Results represent n=8-18 mice/group as mean ± SEM pooled from at least two different experiments.

Bacteria of the family Verrucomicrobiaceae are also absent in most of SPF-2 and SPF-3 mice, while some SPF-7 mice harbour Verrucomicrobiaceae, but in lower relative abundances than in SPF-1 mice (Figure 3.9 C). In contrast, bacteria of the family Prevotellaceae, S24-7, Ruminococcaceae and

unclassified bacteria of the order Clostridales are enriched in mice with lower susceptibility (Figure 3.8 A-B). Bacteria of the family Prevotellaceae, Ruminococcaceae and the order Clostridales spp. are absent or lower abundant in SPF-1 mice compared to SPF-2, SPF-3 and SPF-7 mice (Figure 3.10 A, 3.10 C-D). In contrast, bacteria of the family S24-7 are present in low as well as high abundance in SPF-1 mice and only a small statistically difference exists between susceptible SPF-1 mice and resistant SPF-2, SPF-3 and SPF-7 mice.

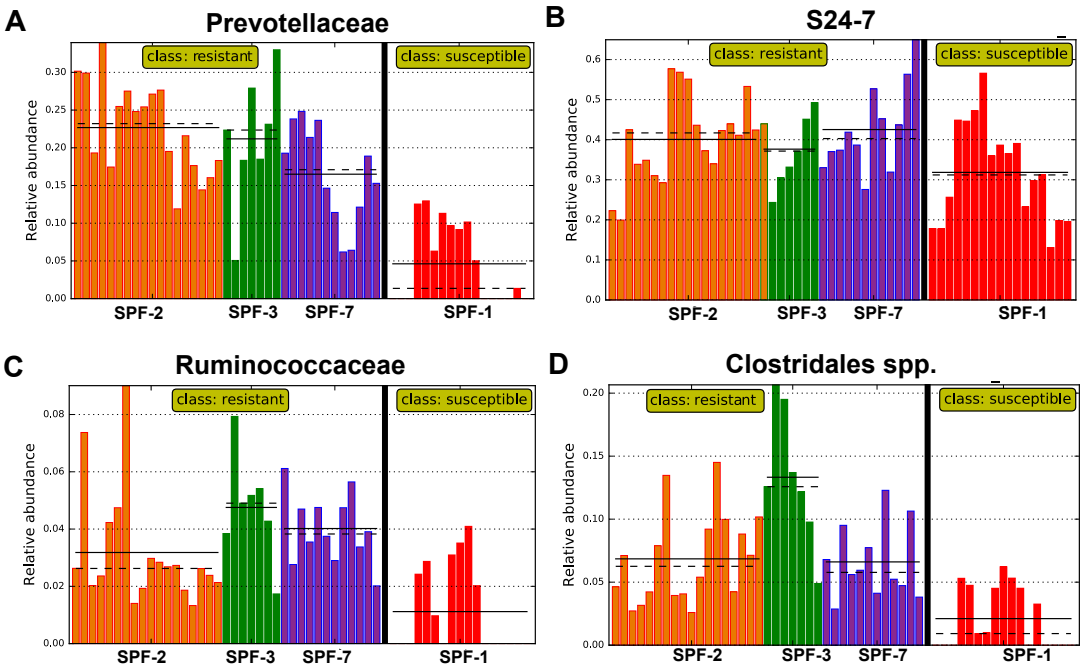

Figure 3.10 - Individual biomarkers associated with higher resistance. Statistically significant differences of microbial signatures linked to higher resistance are displayed of individual mice (SPF-1, SPF-2, SPF-3, SPF-7) on family level before infection: (A) Prevotellaceae, (B) S24-7, (C) Ruminococcaceae, (D) Clostridia spp. Results represent n=8-18 mice/group as mean ± SEM pooled from at least two different experiments.

By using comparative analysis coupled with different microbiota settings and statistically tests, microbial signatures associated with susceptibility and resistance to *C. rodentium* colonization at early time points were identified. Presence of bacteria of the families Verrucomicrobiaceae, Bacteroidaceae and Alcaligenaceae were associated with higher susceptibility, whereas bacteria of the family Prevotellaceae, S24-7, Ruminococcaceae and unclassified bacteria of the order Clostridales were linked to higher resistance.

3.4.4 Cohousing experiments reveal bacterial biomarkers responsible for resistance against *C. rodentium*

To identify if bacteria linked to susceptibility or resistance can be transferred and alter susceptibility, SPF-1 (susceptible) and SPF-2 (resistant) mice were cohoused for 4 weeks and mice were infected with 10^7 CFU *C. rodentium* by oral gavage (Figure 3.11 A).

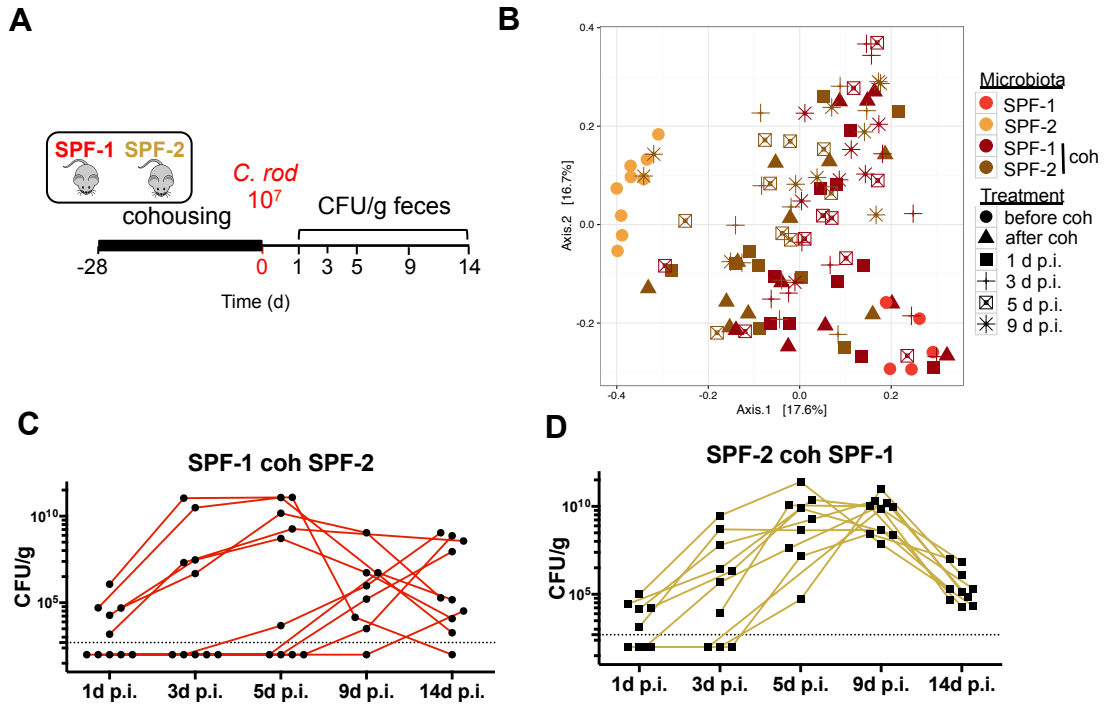

Figure 11 - Altered susceptibility to *C. rodentium* infection upon cohousing of SPF-1 and SPF-2 mice. (A) SPF-1 and SPF-2 mice were cohoused for 4 weeks and infected with *C. rodentium* (*C. rod*). (B) Fecal microbiota was analyzed using 16S rRNA sequencing before and after cohousing as well as during infection, day 1, 3, 5 and 9 post infection (p.i.). β-diversity was analyzed using Bray-Curtis dissimilarity matrix and PCoA plot. (C-D) *C. rodentium* CFUs in feces of individual mice of SPF-1 cohoused (coh) with SPF-2 mice (C) and SPF-2 mice coh with SPF-1 (D) are displayed on day 1, 3, 5, 9 and 14 p.i. Dashed lines indicate the limit of detection. Results represent one independent experiment with n=8-10 mice/group.

Upon cohousing, fecal microbiota of SPF-1 and SPF-2 mice showed a distinct and diverse microbial community, at steady state (after cohousing) and during *C. rodentium* infection. Most of the mice display a mixed community of the original SPF-1 and SPF-2 microbiota (Figure 3.11 B). Interestingly, when SPF-1 mice cohoused with SPF-2 mice (SPF-1 coh SPF-2) are infected with *C. rodentium*, around half of the mice displayed lower CFU numbers at early time points of infection (1d, 3d, 5d p.i.), indicating a higher resistance (Figure 3.11 C). Whereas the other SPF-1 coh SPF-2 mice phenocopy the SPF-1

phenotype (non-cohoused). Notably, also some SPF-2 mice cohoused with SPF-1 mice (SPF-2 coh SPF-1) showed an altered susceptibility. As seen in SPF-1 coh SPF-2, around half of the SPF-2 coh SPF-1 mice were characterized by enhanced susceptibility, whereas the other half mimics a non-cohoused SPF-2 phenotype (Figure 3.11 D). Color-coding of resistant and susceptible mice upon infection in afore analyzed PCoA plot (Figure 3.11 B) did not identify distinct microbial communities in resistant and susceptible mice (Figure 3.12 A).

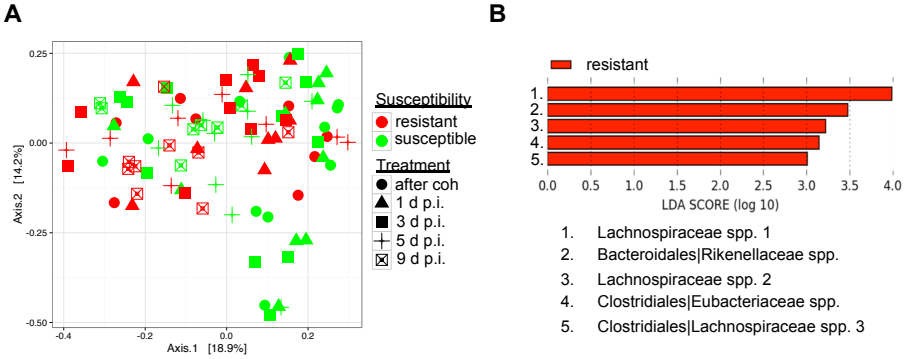

Figure 3.12 - Microbial signature associated with higher resistance after cohousing. SPF-1 and SPF-2 mice were cohoused for 4 weeks and infected with *C. rodentium*. Fecal microbiota was analyzed using 16S rRNA sequencing before and after cohousing as well as during infection, day 1, 3, 5 and 9 post infection (p.i.) (data see Figure 11). (A) β-diversity was analyzed using Bray-Curtis dissimilarity matrix and PCoA plot ordered regarding their susceptibility to *C. rodentium* infection. (B) Statistically significant differences in the fecal microbiota between susceptible and resistant mice before treatment was analyzed on OTU level using LEfSe (Kruskal-Wallis test $p<0.05$, LDA 3.0), ranking them accordingly to their effect size. Results represent one independent experiment with n=8-10 mice/group.

Using LEfSe analysis on OTU level at steady state revealed that specific bacteria of the order Clostridales (Eubacteriaceae spp., Lachnospiraceae spp. 1, Lachnospiraceae spp. 2, Clostridales spp. 3) and of the order Bacteroidales (Rikenellaceae spp.) are linked to resistance (Figure 3.12 B). No bacteria were found to be associated with increased susceptibility. Abundances of statistically different bacteria associated with resistance are displayed per individual mice sorted according to their colonization on 1d p.i. (Figure 3.13). Moreover, relative abundances of statistically different OTUs linked to resistance in cohouse mice were analyzed in SPF-1 and SPF-2 mice at steady state (Figure 3.14). Notably, Rikanellaceae spp. and Lachnospiraceae spp. 3 were not associated with resistance (SPF-2), although displayed higher abundance in susceptible mice (SPF-1). However, Lachnospiraceae spp. 1 and Lachnospiraceae spp. 2 were absent in susceptible SPF-1 mice and

abundant in resistant SPF-2 mice, similar what was seen in cohousing experiments. Therefore, Lachnospiraceae spp. 1 and Lachnospiraceae spp. 2 are valuable bacterial candidates linked to resistance.

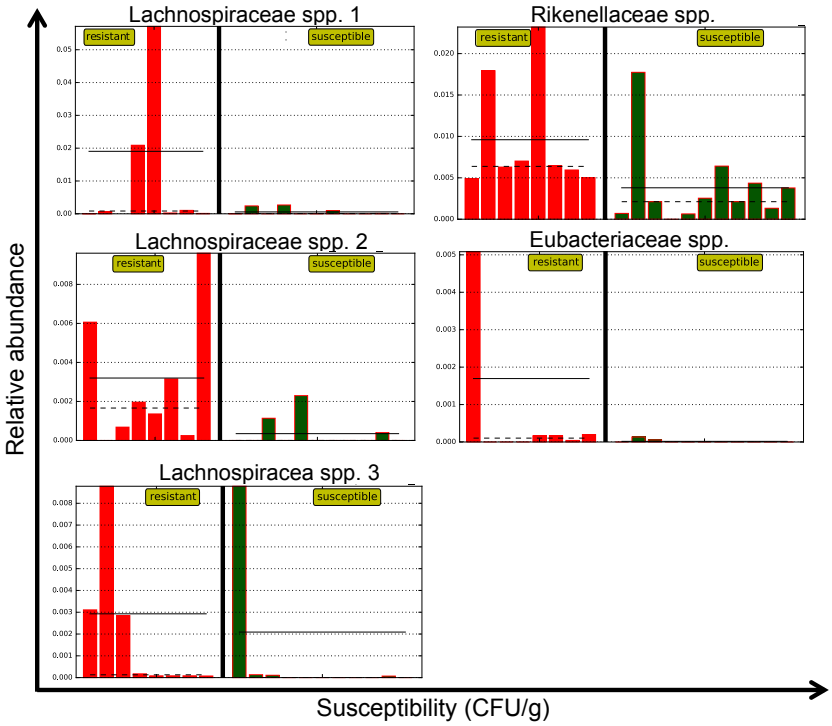

Figure 3.13 - Individual biomarkers linked to higher resistance in cohoused mice. Statistically significant different bacteria between resistant and susceptible mice associated with ameliorated disease are displayed of individual mice before infection and ordered regarding their susceptibility. Results represent n=8-10 mice/group as mean ± SEM from one independent experiment.

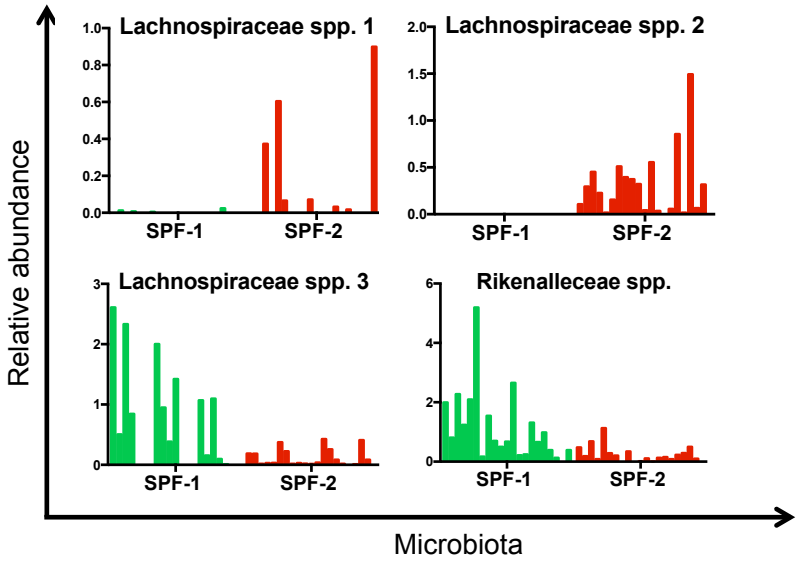

Figure 3.14 - Abundance of identified biomarkers in SPF-1 and SPF-2 mice. Identified bacteria linked to higher resistance were analyzed in SPF-1 and SPF-2 before and during infection on OTU level. Results represent n=5-6 mice/group from one independent experiment.

Moreover, full-length 16S rRNA sequences of the two bacterial candidates associated with resistance were reassembled by using database of de novo assembled 16S rRNA seqeunces (unpublished data). Based on the full-length 16S rRNA sequence, phylogenetic tree was created, including the two bacteria, which are linked to resistance and displayed them with bacteria of the gut bacterial community Altered Schaedler flora (ASF). The analysis revealed that the two candidate bacteria of the family Lachnospiraceae are distinct from each other, likely deriving from different bacterial genera (Figure 3.15).

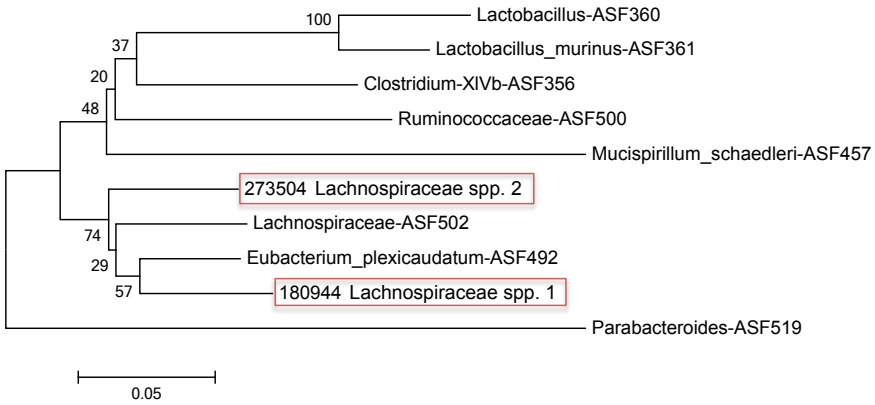

Figure 3.15 - Phylogenetic tree of Lachnospiraceae spp. associated with protected phenotype. Phylogenetic tree from full-length 16S rRNA gene sequences of bacteria of the family Lachnospiraceae associated with resistance are displayed together with the eight bacteria of the Altered Schaedler flora (ASF).

In summary, cohousing experiments with susceptible and resistant mice as well as subsequently, comparative analysis in non-cohoused susceptible (SPF-1) and resistant (SPF-2) mice could reveal that two unclassified bacteria of the family Lachnospiraceae are highly associated with enhanced resistance to *C. rodentium* at early time points of infection.

3.5 Discussion

One of the major functions of the gut microbiota is to defend the host from invading pathogens, which is referred to as colonization resistance. The microbiota not only interacts directly with the pathogen, but also indirectly by activating the immune system and strengthening the epithelial barrier to inhibit pathogen colonization (Buffie and Pamer, 2013). An imbalanced microbiota composition increases susceptibility to infections (Doorduyn et al., 2006; Owens et al., 2008; Sekirov et al., 2008). Already at homeostatic conditions, the composition of the microbiota varies among humans, which could explain differences in susceptibility to infectious diseases. However, which specific intestinal bacteria are able to confer colonization resistance has not been comprehensively addressed.

Citrobacter rodentium is a murine model for pathogens inducing A/E lesion in the gut epithelium such as the human pathogens EHEC and EPEC. The gut microbiota strongly affects the susceptibility to *C. rodentium* infections. For example, it has been shown that antibiotic treatment renders mice more susceptible to *C. rodentium* infections (Wlodarska et al., 2011).

We hypothesized that recent advances in analytical and experimental tools would allow the identification and evaluation of distinct bacterial biomarkers to predict disease susceptibility to *C. rodentium*. First, by using isogenic mouse lines from different breeding facilities, microbial signatures were identified, which are associated with reduced susceptibility to *C. rodentium*. In the next step, cohousing experiments of resistant and susceptible mice confirmed specific bacteria associated with higher disease protection.

C57BL/6N isogenic mouse lines from different breeding facilities were studied for differences in their microbiota composition. Distinct microbiota composition in each breeding facility could be identified. Differences in the microbiota composition were already observed at phylum level, e.g. ratio of Firmicutes to Bacteroidetes. Interestingly, mouse lines derived from the same breeding facility, but from different barriers, displayed a similar microbiota composition than mice from different breeding facilities. In addition, studied C57BL/6N mouse lines showed different kinetics of *C. rodentium* infection. For example, SPF-1 mice displayed higher luminal colonization of *C. rodentium* than SPF-2,

SPF-3 and SPF-7 mice at early time points (day 1 and 3 p.i.), whereas at later time points (9 p.i. and 14 d p.i.) SPF-1 mice started to clear the infection and showed lower luminal abundance of *C. rodentium* than SPF-2 mice.

We identified that distinct features of the intestinal microbiota are associated with lower *C. rodentium* colonization in early phase of infection. Specifically, bacteria of the families Prevotellaceae, Ruminococcaceae, and S24-7 as well as unclassified bacteria of the order Clostridiales were positively correlated with higher resistance to *C. rodentium* infection. To exclude that different disease susceptibilities are explained by genetic differences reported in C57BL/6 mice due to different C57BL6/N sub-lines or acquired during inbreeding by spontaneous mutation, one susceptible (SPF-1) and resistant (SPF-2) mouse line were cohoused with germ-free C57BL/6NTac mice. Fecal microbiota composition as well as disease progression was highly similar in formerly germ-free mice as their respective cohousing partner, indicating that the phenotype is fully microbiota dependent. If potential genetic differences in other isogenic C57BL/6N lines could contribute to varying colonization susceptibility has to be evaluated. By using cohousing experiments of protected and susceptible mice, we identified different sets of bacteria associated with lower colonization: specific bacteria of the order Clostridiales (Eubacteriaceae and Lachnospiraceae) and of the order Bacteroidales (Rikanellaceae). Interestingly, two bacteria of the family Lachnospiraceae were also higher abundant in non-cohoused SPF-2 than non-cohoused SPF-1 mice and therefore, represent strong candidates for conferring resistance to *C. rodentium* infection. Lachnospiraceae (phylum Firmicutes, class Clostridia, order Clostridiales) are a bacterial family in the gastrointestinal tract of many mammalians and include bacteria, which are strictly anaerobe (Gosalbes et al., 2011; Kittelmann et al., 2013). Some bacteria of the family Lachnospiraceae are butyric acids producer, a short chain fatty acid (SCFA), arising from microbial fermentation (Meehan and Beiko, 2014). Butyrate is a crucial energy source for other bacteria and colonic epithelial cells (Meehan and Beiko, 2014; Pryde et al., 2002). Interestingly, patients with inflammatory bowl disease exhibit a lower abundance of Lachnospiraceae (Clostridium subsets IV and XIVa).

Isolation of the two bacteria belonging to the family Lachnospiraceae that were associated with protection against *C. rodentium* is essential to characterize bacteria in more detail as well as to identify the contribution to confer colonization resistance. Moreover, transfer of isolates to susceptible mice is crucial to validate the association with protection.

Strikingly, another study correlated resistance to *C. rodentium* infection with a decrease in bacteria of the order Clostridiales in NIH Swiss mice. (Willing et al., 2011). However, it has to be evaluated, if the transfer of the bacteria of the family Lachnospiraceae from our study also confers resistance in other mouse lines with different genetic backgrounds than seen in C57BL/6N (e.g. C3H/HeJ, NIH Swiss).

An important barrier to inhibit enteric pathogen colonization is constituted by the host epithelium and the associated mucus layer. For example, greater numbers of adherent *C. rodentium* attached to the cecum is higher in mice lacking a mucus layer (*Muc2*$^{-/-}$ mice) compared to wild type animals (Bergstrom et al., 2010). Mucus not only forms a physical barrier and reservoir for antimicrobial peptides, it also forms a distinct niche for commensal bacteria that use mucins and associated molecules as carbon sources (Johansson et al., 2011). Members of the order Clostridiales are localized in close proximity to the gut epithelium within the mucus layer and have been suggested to be critical regulators of the turnover of the mucus layer. Previous studies have indicated that bacteria of the order Clostridiales, i.e. *Clostridium septicum*, *Clostridium perfringens*, show mucolytic activity and metabolize mucus-derived oligosaccharides (Deplancke et al., 2002; Macfarlane et al., 2001). Anaerobic fermentation of carbohydrates of the mucus layer by these bacteria results in production of SCFAs, which in turn increase mucus production and secretion (Burger-van Paassen et al., 2009; Finnie et al., 1995). Similarly, treatment with the phytonutrient eugenol has been reported to confer resistance to *C. rodentium* infection by increasing inner mucus thickness (Wlodarska et al., 2015). In the same study eugenol treatment additionally augments abundances of bacterial families within the Clostridiales (Wlodarska et al., 2015). Therefore, mucus thickness has to be addressed in our analyzed isogenic mouse lines, which may explain varying susceptibilities to *C. rodentium* infections. Moreover, cohousing experiments of protected and

susceptible mice could reveal if specific transferred bacterial features (e.g. Lachnospiraceae) strengthen mucus barrier.

Moreover, antibiotic treatment was shown to deplete butyrate-producing Clostridia, which enhance tissue oxygen allowing the expansion of aerotolerant *Salmonella* (Rivera-Chávez et al., 2016). If Lachnospiraceae are also important for preventing *C. rodentium* by lowering luminal oxygen level has to be addressed in the future.

Besides the maintenance of the epithelial barrier, another key factor for conferring resistance against pathogen colonization is competition for essential nutrients and niches. For example, *C. rodentium* can be outcompeted by other γ-Proteobacteria, such as *E. coli*, which is accumulated in the gut upon infection (Lupp et al., 2007) and may occupy niches and compete for nutrients. Colonization of *E. coli* in germ-free mice reduces burden of *C. rodentium,* whereas *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* do not outcompete *C. rodentium* (Kamada et al., 2012). Under normal conditions, *B. thetaiotaomicron* can catabolize mono- and polysaccharides. However, if mice are fed a simple diet of monosaccharides, *B. thetaiotaomicron* reduces *C. rodentium* burden by competing for available monosaccharides (Kamada et al., 2012). Strikingly, SPF-6 mice were the only studied mouse line showing abundances of commensal *E. coli*, which was decreased over time during infection excluding that in our microbiota settings *E. coli* is blooming during *C. rodentium* infection and confers resistance. It cannot be excluded and it is highly likely that other not yet identified commensal bacteria compete for nutrient availability with *C. rodentium* and therefore, conferring colonization resistance directly.

Not only competition of nutrients influence pathogen colonization, but also metabolites derived from commensal bacteria can be exploited by the pathogen for enhancing virulence expression and proliferation. It has been reported that colonization of *B. thetaiotaomicron* resulted in elevated concentrations of succinate, which in turn, enriches expression of virulence genes of *C. rodentium*, e.g. genes important for A/E lesions, and therefore enhances pathogenicity (Curtis et al., 2014).

Moreover, abundance of segmented filamentous bacteria (SFB) was observed to differ in isogenic C57BL/6 mice from different vendors. Increased

SFB levels have been reported to correlate with augmented resistance to *C. rodentium* via stimulation of $CD4^+$ T helper cells and therefore, amplified production of IL-17/IL-22-RegIIIγ-axis (Ivanov et al., 2009). In our study, by using 16S rRNA sequencing, abundance of SFB was not different in susceptible (SPF-1) and resistant (SPF-2) mice. However, these data should be verified by using a SFB specific PCR.

Translation to human research is important to generate new mucosal therapies. *C. rodentium* is a murine pathogen, which is used to mimic human pathogenicity of EHEC and EPEC in mice. *C. rodentium* possesses characteristic pathogenic features of EHEC and EPEC such as the formation of A/E lesions, including similar virulence factors, e.g. type III effector protein Tir and the bacterial outer membrane protein intimin (Petty et al., 2010; Schauer and Falkow, 1993). A/E lesions on the gut epithelium are crucial for proper colonization and virulence (Deng et al., 2003). In contrast to *C. rodentium* and EPEC, EHEC expresses additionally a shiga toxin (stx), which is responsible for intestinal damage and life-threatening systemic diseases such as renal damage (Nataro and Kaper, 1998). Therefore, EHEC have been responsible for severe outbreaks of diarrheal illness (Frank et al., 2011; Rangel et al., 2005). Since stx is absent in *C. rodentium*, the pathogen does not cause the same pathogenicity in mice as EHEC does in humans. Vice versa, infection with EHEC in mice does not recapitulate formation of A/E lesions or intestinal damage (Mohawk and O'Brien, 2011). To overcome these limitations and generate an improved model to study EHEC pathogenicity Mallik and colleagues have been developed an stx-producing *C. rodentium* strain ($\Phi stx2_{dact}$) combining the ability to cause A/E lesions and stx-mediated inflammatory damage (locally and systemically) (Mallick et al., 2012). Evaluating the impact of the intestinal microbiota composition as well as the identified microbial signatures to stx-producing *C. rodentium*, which develop in mice pathogenicity similar to human EHEC infection, could provide valuable insights into the interplay of microbiota and EHEC infections in the future.

In summary, by using isogenic mouse lines from different breeding facilities, we demonstrated that each mouse line displays a distinct microbiota composition, featuring different susceptibilities to *C. rodentium*. We could identify bacterial signatures associated with lower colonization of *C. rodentium*

at early time points of disease, such as two bacteria of the family Lachnospiraceae, representing novel and uncultured bacterial species. Proof of their protective properties and an understanding of how those bacteria confer resistance are still lacking. Therefore, isolation of the bacterial strains is a necessary step to validate the association and enable functional studies in the future.

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Abbreviations

A/E	Attaching/effacing
ANOVA	Analysis of variance
ASF	Altered Schaedler flora
BSL	Baseline
CFU	Colony forming units
Coh	Cohoused
C. rod	*Citrobacter rodentium*
EDTA	Ethylendiamintetraacetat acid
EHEC	Enterohaemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *E.coli*
GI	Gastrointestinal tract
GF	Germ-free
Lac	Lactose-fermenting
LDA	Linear discriminant analysis
LEE	Locus of enterocyte effacement
LEfSe	LDA effect size
LB	Lysogeny broth
NaCl	Sodium chloride
NIH	National Institutes of Health
o.g.	Oral gavage
OTU	Operational taxonomic level
PBS	Phosphate-buffered saline
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
p.i.	Post infection
Reg3	Regenerating islet-derived protein
SCFA	Short chain fatty acid
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SFB	Segmented filamentous bacteria
SPF	Specific pathogen free
Stx	Shiga toxin
T3SS	Type III secretion system

TE	Tris EDTA
Tir	Translocated intimin receptor
Tris	Tris(hydroxymethyl)-aminomethan

4 CHAPTER

General conclusion and outlook

More than 100 trillion microorganisms from estimated at least 1000 microbial species including bacteria, archaea, fungi and protozoans as well as their respective viruses colonize the human body (Qin et al., 2010). The collection of microbial populations found on a host is referred to as microbiota and form complex ecosystems. Microbes within these ecosystems are strongly interacting with each other, but also individually or as community with the host. The microbiota coevolved with the host and contributes to many physiological function of the host. It influences the development and regulation of the host immune system and protects the host against invading pathogens (Clemente et al., 2012). The microbiota mediates resistance via several distinct ways: Directly by competing for nutrients and ecological niches, indirectly or immune-mediated by strengthening the epithelial barrier and by inducing protective immune responses (McKenney and Pamer, 2015). Alterations in the microbiota composition may disturb the delicate host-microbiota balance negatively impacting the host. Distinct microbial alterations have been associated to a broad range of human diseases such as obesity, type 2 diabetes, inflammatory bowl disease and neurodevelopmental disorders (Wu et al., 2015). But even in homeostatic conditions, the microbial composition of the microbiota differs strongly among humans (Lozupone et al., 2012). Besides genetic variations, environmental factors such as diet, antibiotic treatment and hygiene have a great impact on the microbiota composition (Round and Mazmanian, 2009). This could explain varying susceptibilities to enteric infections in humans. Indeed, antibiotic treatment has been associated with augmented susceptibility to enteric infections e.g.

Clostridium difficile and non-typhoidal *Salmonella* disease (Doorduyn et al., 2006; Owens et al., 2008; Sekirov et al., 2008).

By using germ-free mice and conventionally raised mice treated with different antibiotics, researchers have identified long time ago the importance of the microbiota in mediating resistance to enteric infections (Bohnhoff and Miller, 1962; Carter and Collins, 1974). However, research has since then largely focused on studying in detail pathogenic bacteria rather than beneficial gut bacteria. Only in the last decade, culture-independent high-throughput sequencing technologies have revealed the complexity of commensal gut bacteria and resulted in a shift of the focus of many microbiologist on studying intestinal bacteria and their interactions with the host and pathogens. However, by using high-throughput sequencing researchers associate bacterial communities to specific diseases rather than showing direct causality. Therefore, little is known about effects of individual members of the microbiota on enteric pathogens and host immune responses. However, this is crucial to develop mucosal therapeutics based on defined communities to reconstitute the intestinal ecosystem. Already now, transfer of whole communities derived from the stool of healthy persons to diseased patients offers an effective and therapeutic approach. For example, reconstitution of the microbiota by fecal transplantation is successfully used to cure recurrent *C. difficile* infection with an efficiency of over >90% (Bakken, 2009; van Nood et al., 2013).

Although fecal transplantation is cost-efficient and effective, transfer of bulk bacterial communities harbors several risks factors and therefore, high regulatory hurdles. An example of the risk is the transfer of opportunistic bacteria or not yet identified pathogenic bacteria that are potentially harming the recipient or have unwanted consequences. Currently, the fecal transplant donor is screened before fecal transplantation to identify currently known pathogens, but because of the high costs associated the complete community is not screened in detail routinely. Therefore, the outcome and long-term success of fecal transplantation in recipients has not been assessed thoroughly. Moreover, the colonization and composition of the transferred community is based on host genetics of recipients and environmental factors such as diet and medical treatments, potentially causing different immune

responses in recipients than in donors. Therefore, it is of high interest to study intestinal bacteria, their interaction with other gut bacteria and pathogens as well as the activation of immune responses in disparate host genotypes. Administrating fully defined and reproducible mixtures of beneficial bacteria will be a more safe approach to treat enteric infections and also would simplify the approval process for bacterial transplantation.

The main aim of the presented works was to identify intestinal bacteria contributing to enhanced resistance towards enteric pathogens as well as to characterized novel microbiota-modulated protective immune responses. To this end, C57BL/6N mice from different breeding facilities were used, which featured distinct microbiota compositions as demonstrated using 16S rRNA sequencing. Isogenic C57BL/6N mouse lines had different susceptibilities to the enteric murine pathogens *Salmonella enterica* spp. serovar Typhimurium and *Citrobacter rodentium*. Cohousing mice from different breeding facilities with isogenic germ-free C57BL/6NTac mice excluded any influence of the genotype on this phenotype and corroborated that differences seen in mice from different breeding facilities are microbiota-dependent. In both infection models microbial signatures correlating with increased resistance were identified, which differed in *S.* Typhimurium and *C. rodentium* infection.

In case of *C. rodentium*, statistical analysis of 16S rRNA sequencing data revealed that two bacterial species of the family Lachnospiraceae were linked to lower susceptibility to *C. rodentium*. These results are currently only based on culture-independent approaches, enabling to establish a strong association, but no direct causality. Therefore, isolation of bacterial candidates will be crucial to validate the association and to identify in more detail the mechanisms involved in conferring resistance. As next steps, metabolomic profiles of the two bacterial species could help to identify direct nutrient interaction with *C. rodentium* inhibiting pathogen growth *in vivo*. Moreover, it has been reported that higher mucus thickness lowers susceptibility to *C. rodentium* (Bergstrom et al., 2010). In turn, properties of the mucus barrier are dependent on the microbiota composition (Jakobsson et al., 2015). It would be interesting to characterize if the two bacteria of the family Lachnospiraceae affect mucus barrier thickness and therefore, indirectly lower susceptibility.

In case of *S.* Typhimurium infection, bacteria of the families S24-7 and Prevotellaceae as well as the family Verrucomicrobiaceae were associated with increased resistance. Transfer of 11 bacterial strains, cultured from feces of resistant mice, was able to phenocopy the resistant phenotype in previously susceptible mice. Cultured bacterial strains included members of the families Prevotellaceae, Bacteroides, S24-7, Lactobacillaceae and Lachnospiraceae. However, it is unclear if a single bacterial strain or the complete community is responsible for conferring resistance. In addition, the stability of transferred bacterial strains in the intestinal microbiota conferring resistance has to be addressed. Moreover, in this study microbiota-induced interferon (IFN) γ, produced by $CD4^+$ T cells in the LP was identified as one novel key factor for controlling *Salmonella* loads in the mucosal tissue. IFNγ signaling has been reported to activate different microbicidal mechanisms enhancing killing of infected macrophages and augment T cell responses (Foster et al., 2003; Gordon et al., 2005; Kaiser et al., 2012; Nairz et al., 2008). In addition, Interferon-γ has been described to contribute to mucus excretion by goblet cells of *Salmonella*-infected mucosa (Songhet et al., 2011). Downstream pathways of microbiota-induced IFNγ, which are involved to lower pathogen loads should be studied in the future.

In both studies, *in vivo* experiments with mice were used to ensure similar genotypes as well as environmental conditions such as diet and medical treatments allowing functional and mechanistic research. Mice and humans share a similar physiology and immunological features. Therefore, animal models are increasingly used to study functional as well as mechanistic research in a controlled experimental defined setup. Especially in the microbiota research, mouse models are widely used because of simple manipulation and intervention of the microbiota as well as relatively low maintenance cost and short life cycle. Moreover, animal experiments have the advantage to collect easily samples before and during disease as well to manipulate simply the microbiota to admit assessing causality in microbiota-associated diseases. Nevertheless, the human and murine microbiota show differences in their relative abundances of intestinal bacteria (Nguyen et al., 2015; Xiao et al., 2015). However, high similarity at the functional level of the

murine microbiota to its human counterpart was observed using metagenomic analysis (Xiao et al., 2015). In most of the cases, human studies have the limitations to establish only correlations but not causality. Currently, also human microbiota-associated (HMA) mouse models have been started to be used. To establish HMA mice, fecal stool samples from humans are transferred to germ-free mice. By transferring the human microbiota, several human diseases could be modeled in mice (Arrieta et al., 2015; Koren et al., 2012; Ridaura et al., 2013). However, if HMA mice are a good tool to study infectious diseases has to be evaluated in the future as mice and humans differ in several characteristics, which makes translational research from mouse to human challenging.

In summary, identification and isolation of specific members of the microbiota that are conferring resistance is essential for engineering the microbiota in the future. Transferring stable, well-characterized bacterial communities would offer an effective and cost-efficient method to lower susceptibility to enteric infections. In addition, a well-studied bacterial community allows minimizing the transfer of potential harmful commensal bacteria.

In the meantime, collecting stool samples from healthy patients, identifying their microbiota composition and storing them in a stool bank could simplify screens for potential donors as to eventually transfer only material from donors with similar genetic and environmental (e.g. diet, medical treatments) backgrounds to enhance stability of the transferred bacterial community. Until now, identifying in detail a complete fecal microbiota and the genotype of the host in a cost-efficient way is challenging.

In summary, the microbiota has a high potential as therapeutic target for enteric infections, but also for other common diseases such as obesity, diabetes and inflammatory bowl disease. While we and others provided novel insights into the complex interplay between the microbiota and the host, there is still a long way to go before distinct intestinal bacteria can be used as a safe and effective drug.

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Abbreviations

CD	Cluster of differentiation
HMA	Human microbiota-associated
IFN	Interferon

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- Study abroad program Erasmus (Italy)

PUBLICATIONS

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Błażejewski A. J., **Thiemann S.,** Schenk A., Pils M. C., Gálvez E. J. C., Roy U., et al. (2017). Microbiota Normalization Reveals that Canonical Caspase-1 Activation Exacerbates Chemically Induced Intestinal Inflammation. Cell Reports, 19(11), 2319–2330.

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Heine, W., Beckstette, M., Heroven, A. K., **Thiemann, S.,** Heise, U., Nuss, A. M., et al. (2018). Loss of CNFY toxin-induced inflammation drives Yersinia pseudotuberculosis into persistency. PLoS Pathogens, 14(2), e1006858

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Thiemann S., Osbelt L., Galvez E., Lesker TR, Strowig T., (Under preparation). Identifying microbial signatures associated with reduced susceptibility to *Citrobacter rodentium* infection.

PRESENTATIONS

Thiemann S, Galvez E, Strowig T (2015) Deconstruction of direct and indirect colonization resistance during Salmonella infection, Poster presented at Gut Microbiota Modulation of Host Physiology: The Search for Mechanism, Keystone Resort, Colorado, USA

Thiemann S, Galvez E, Strowig T (2015) Isogenic and isobiotic mouse lines reveal microbiota-mediated indirect colonization resistance against Salmonella infection, Presentation at 9th Seeon Conference, Germany